The Human Papillomavirus Type 16 E6 Oncogene Regulates microRNA-218 via the Histone Acetyltransferase p300 in Cervical Carcinoma Cells

by

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THE HUMAN PAPILLOMAVIRUS TYPE 16 E6 ONCOGENE REGULATES MICRORNA-218 VIA THE HISTONE ACETYLTRANSFERASE P300 IN CERVICAL CARCINOMA CELLS

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University of Pittsburgh, 2010

Human papillomaviruses (HPVs) are involved in the pathogenesis of different types of human cancers, especially cancer of the cervix (CaCx). MicroRNAs (miRNAs) are post-transcriptional gene regulators that have recently been associated with many types of cancers. We analyzed the expression of cellular miRNAs in HPV-16 positive cervical cell lines and tissues via microarray, Northern blotting, and quantitative RT-PCR. Three miRNAs were overexpressed and 24 underexpressed in cervical cell lines containing integrated HPV-16 DNA compared to the normal cervix. An HPV-negative CaCx cell line, C-33A, showed underexpression of four miRNAs compared to the normal cervix. Also, nine miRNAs were overexpressed and only one underexpressed in cell lines containing integrated HPV-16 DNA compared to C-33A. MicroRNA-218 (miR-218) was specifically underexpressed in cell lines, cervical lesions and cancer tissues containing integrated HPV-16 DNA compared to both C-33A and the normal cervix. Exogenous expression of the HPV-16 E6 oncogene reduced miR-218 expression, and conversely, RNA interference of E6/E7 oncogenes in an HPV-16 positive cell line increased miR-218 expression. Furthermore, exogenous expression of miR-218 in HPV-16 positive cell lines decreased expression of the epithelial-specific gene *LAMB3,* which is involved in cell migration and tumorigenicity.

We analyzed the expression of cellular miRNAs in additional clinical samples including six HPV-16 positive cervical cancers, three cervical dysplasias, and four normal cervical tissues using TaqMan® MicroRNA Arrays V2.0. Eighteen miRNAs were overexpressed and two underexpressed in cervical cancer tissues compared to normal cervical tissues. Nine miRNAs were consistently overexpressed, and two miRNAs were consistently underexpressed, including miR-218, in cervical dysplasias and cervical cancer tissues compared to normal tissues. We also found that exogenous expression of miR-218 in HPV-16 positive cell lines decreased expression of the extracellular matrix protein MMP3, which is involved in the epithelial-mesenchymal transition.

We also demonstrated that HPV-16 E6 regulates miR-218 via the histone acetyltransferase p300 in cervical cells. The expression of miR-218 was found to be reduced in HPV-negative cells upon p300 knock-down or p300 inhibition with anacardic acid, and expression of miR-218 was reactivated in HPV-16 positive cells upon EZH2 knock-down or inhibition with adenosine dialdehyde. Reactivation of miR-218 was enhanced upon treatment with a combination of 5-azacytidine, trichostatin A, and adenosine dialdehyde. We also demonstrated that miR-218 reduces the migration and invasion of SiHa cervical cancer cells, indicating that miR-218 functions as a tumor suppressor in cervical cancer and that it may have therapeutic value.

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1.0 CHAPTER 1

GENERAL INTRODUCTION

1.1 HUMAN PAPILLOMAVIRUS BIOLOGY

Human papillomaviruses (HPVs) are common papilloma (wart)-causing viruses of the Family *Papillomaviridae*. They are responsible for inducing a variety of cutaneous and mucosal lesions and importantly are the cause of cervical cancer (Walboomers et al., 1999) (Greenlee et al., 2001). In addition, recent molecular epidemiologic studies have shown a strong correlation between oncogenic HPV infections and a subset of oropharyngeal cancers (Gillison et al., 2000) (Wong and Munger, 2000). HPV DNA can be found in 99.7% of cervical cancers and 25% of oropharyngeal cancers (Greenlee et al., 2001) (Gillison et al., 2000). Although prophylactic vaccines have been approved by the FDA, cervical cancer remains a threat for the millions of women infected with genital HPVs. The current vaccines protect against two high-risk HPVs (types 16 and 18), which account for approximately 70% of cervical cancers. The length of protection offered by the vaccines is currently unknown.

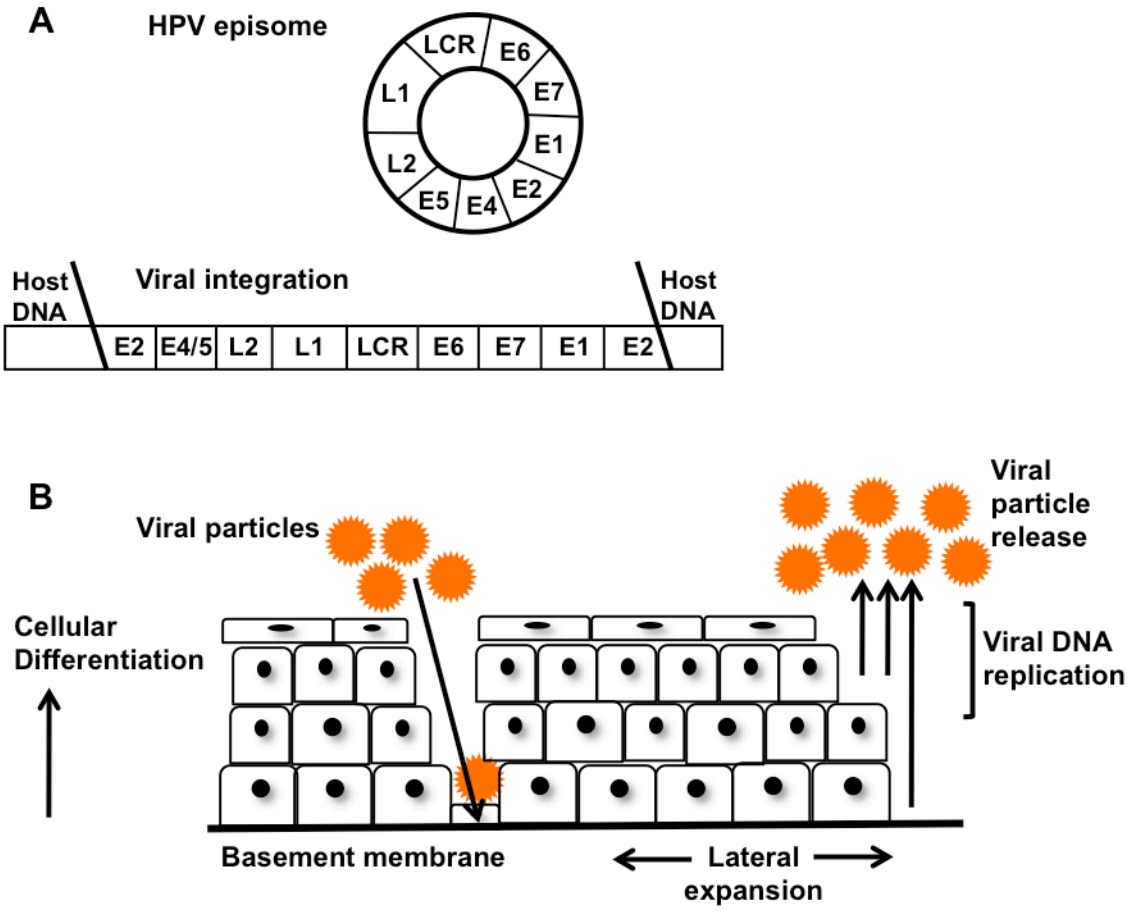
More than 100 HPV types exist, and they can be grouped as low-risk (LR) or high-risk (HR) based on the risk of malignant transformation (de Villiers et al., 2004). LR-HPVs, such as HPV-6 and 11, are associated with epithelial lesions, while HR-HPVs, such as HPV-16 and 18, are associated with cancer (de Villiers et al., 2004). HPVs are small circular non-enveloped double-stranded (ds) DNA viruses (Hebner and Laimins, 2006). The ~8000 base pair (bp) genome of HPVs encodes eight open reading frames (ORFs), which are designated as either early (E) or late (L) according to their temporal expression (Hebner and Laimins, 2006). Polycistronic transcripts are

processed by alternative splicing and translated by ribosomal scanning (Watt, 1998). Early in infection, the non-structural genes E1, E2, E4, E5, E6, and E7 are expressed (Watt, 1998). The E1 helicase and E2 transcription factor cooperate to mediate efficient viral replication, and the E6 and E7 oncogenes work synergistically to deregulate cell cycle controls (Watt, 1998). Viral genomes are maintained episomally in low-copy numbers at this stage (Watt, 1998). Upon terminal differentiation, the late structural genes L1 and L2 are expressed, coinciding with a burst of viral replication and transcription, and virions may then be shed and infect additional tissues (Watt, 1998).

The E6 protein interacts with the tumor suppressor protein p53 (Werness et al., 1990) as a tripartite complex with the E6-associated protein (E6AP) (Huibregtse et al., 1991). This interaction results in ubiquitination and degradation of p53 (Scheffner et al., 1990). E7 interacts with the tumor suppressor protein Rb, preventing its interaction with E2F and thereby allowing constitutive expression of E2F-mediated genes (Chellappan et al., 1992). Persistent infections with HR-HPVs, such as HPV-16, are often characterized by integration of the viral genome into the host chromosomes, which results in increased expression of the E6 and E7 oncogenes due to interruption of the E2 regulatory gene. Integration is thought to occur at fragile sites in chromosomes (Thorland et al., 2003) and may be a consequence of HR-HPV E6/E7-induced chromosomal instability. The disruption or amplification of centrosomes occurs in most cancers, making chromosomal instability a hallmark of cancer. HR-HPV E6 and E7 can cooperate to induce centrosome abnormalities and genomic instability (Duensing et al., 2000). Although HR-HPV E6 and E7 are sufficient to immortalize human keratinocytes in vitro (Munger et al., 1989), additional factors are required for progression to cancer.

The activation of oncogenes such as *H-ras* and the expression of the catalytic subunit of telomerase (*hTERT*) are also required for transformation of primary human epithelial cells (Hahn and Weinberg, 2002).

Figure 1. Human Papillomavirus Organization and Life Cycle. (A) Episomal HPV DNA. In most cases of cervical cancer, the virus integrates into the host DNA. (B) Upon microabrasion, HPV particles infect the basal cells of the epithelium. As the cells divide, the infection expands laterally. Viral early genes are expressed and the genomes replicate. One daughter cell migrates into the suprabasal layer and undergoes differentiation, during which the virus enters the productive phase of its life cycle. Viral DNA replication/amplification, late gene expression, and capsid protein assembly occur. Viral particles are released at the surface of the epithelium (Woodman et al., 2007).



1.2 HUMAN PAPILLOMAVIRUS E6/E7 ONCOGENES AND CERVICAL CANCER

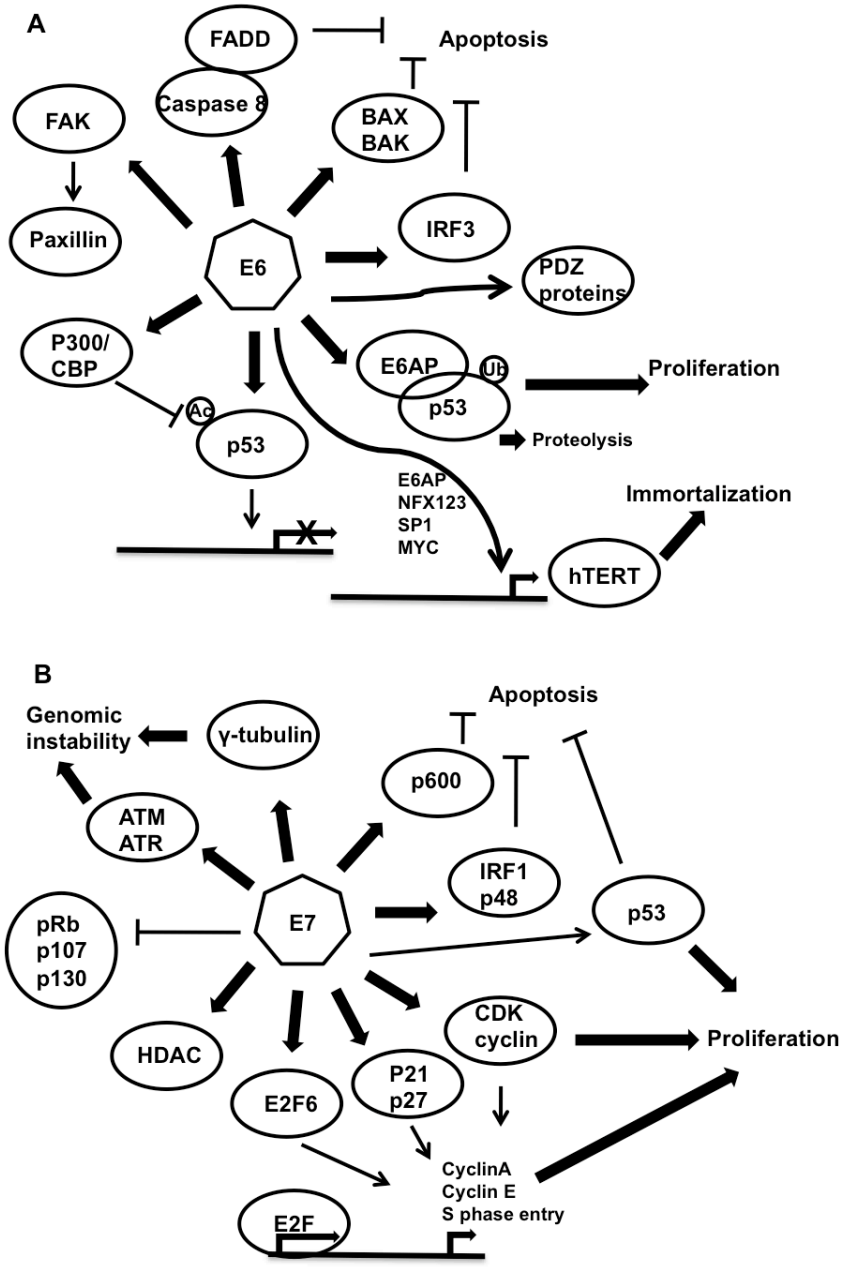
Cervical cancer is the second-highest cancer-related cause of death among women worldwide, with an estimated yearly mortality of 250,000 (Walboomers et al., 1999). In the United States, there are approximately 750,000 new cases of HPV infection every year, including about 4,000 cases of invasive cervical cancer, resulting in nearly 5,000 deaths per year. More importantly, there are approximately 2 million cases of premalignant HPV lesions annually. In general, 5-40% of sexually active teenagers and adults harbor genital HPV infections, depending on the population group. HPVs represent the most common sexually transmitted disease in the United States. It is estimated that more than 50% of all sexually active women in the U.S. will be infected with HPVs at some point during their lifetime.

HPV-16 E6 contributes to cellular transformation through various protein interactions. The E6 protein binds p53 (Werness et al., 1990) in a tripartite complex with E6AP (Huibregtse et al., 1991). This results in the ubiquitination and degradation of p53 (Scheffner et al., 1990). E6 binding of p300/CBP prevents p53 acetylation, (Patel et al., 1999) (Zimmermann et al., 1999), further preventing p53-mediated transcription and increasing proliferation. Interaction with E6AP and transcription factors NFX123, Sp1, and Myc activates hTERT expression, preventing telomere shortening and promoting immortalization (Howie et al., 2009). Degradation of PDZ proteins induces hyperplasia through loss of cell polarity (Nguyen et al., 2003) (Thomas et al., 2008). Degradation of pro-apoptotic protein BAX and BAK inhibits apoptosis (Garnett et al., 2006), as does

interaction with FADD and Caspase 8 (Filippova et al., 2004) (Garnett et al., 2006). Binding to focal adhesion kinase (FAK) and paxillin promote anchorage-independent growth (McCormack et al., 1997) (Tong and Howley, 1997).

HPV-16 E7 bypasses G1-S arrest and induces proliferation through interaction and inhibition of pRB family members and constitutive activation of E2F-responsive genes (Dyson et al., 1989) (Chellappan et al., 1992). E7 also inhibits cyclin-dependent kinase inhibitors p21 and p27, stimulating cyclins and activating CDK2 (Zerfass-Thome et al., 1996) (He et al., 2003). E7 can bind HDACs on some promoters to repress transcription and facilitate HDAC removal at others to activate transcription (Brehm et al., 1998) (Brehm et al., 1999). E7-induced DNA damage activates the ATM-ATR pathway leading to genomic instability (Moody and Laimins, 2009).

Figure 2. Human papillomavirus E6/E7 interactions. (A) Cellular protein interactions of HPV-16 E6. (B) Cellular protein interactions of high-risk HPV-16 E7. (Moody and Laimins, 2010)



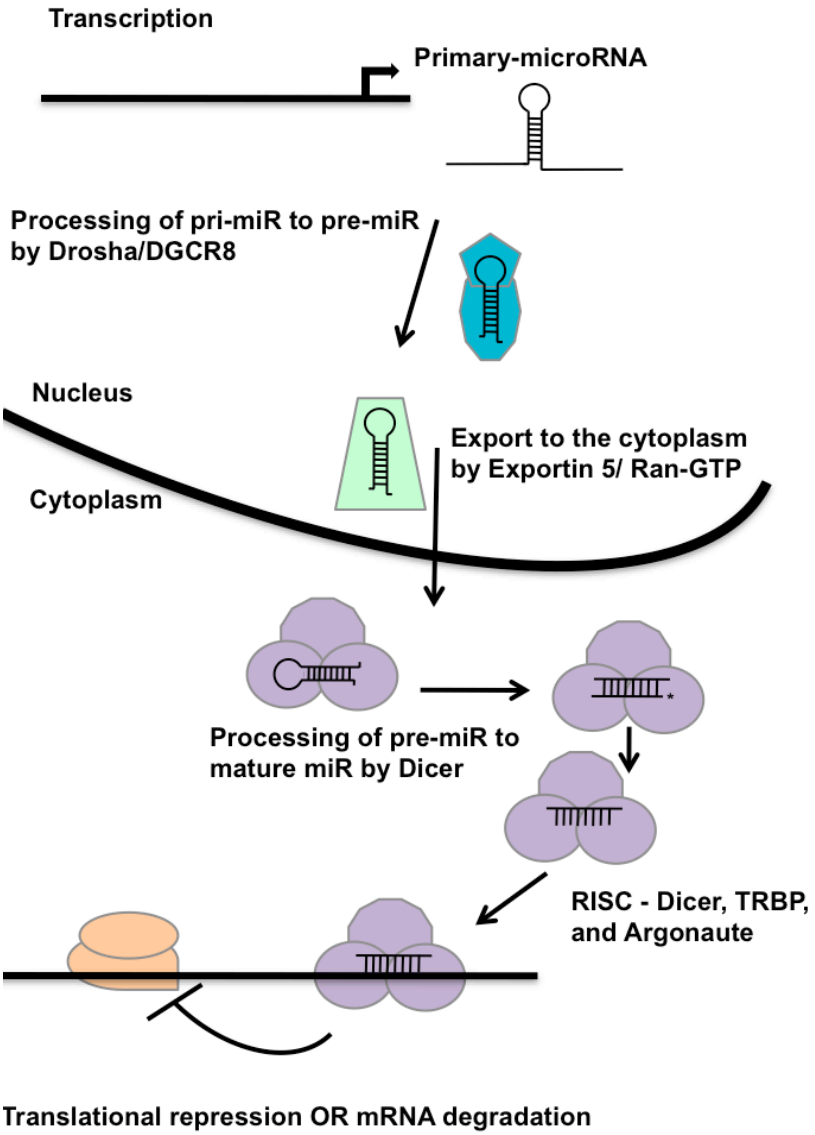
1.3 MICRORNA BIOLOGY

MicroRNAs are a recently characterized class of gene regulators. Mature miRNAs are ~22 nt single-stranded non-protein-coding RNAs that negatively regulate their targets. MiRNAs function by binding to the 3' UTRs of target messenger (m)RNAs thereby mediating either translational repression or mRNA destruction. MiRNAs were first discovered in *C. elegans* (Lee et al., 1993). They have since been found to be conserved across many species, and may regulate thousands of targets via the RNAi pathway (Lewis et al., 2005).

Most miRNAs are transcribed by RNA polymerase II, have a Cap, and are polyadenylated (Lee et al., 2004) (Cai et al., 2004). They are often processed from polycistronic transcripts (Lee et al., 2002). Following transcription, the large primary-miRNA transcripts are processed into precursor-miRNAs by the protein Drosha (Lee et al., 2003). Pre-miRNAs are hairpin-like structures with characteristic 2 nt 3' overhangs. They are exported to the cytoplasm by exportin 5 (Lund et al., 2004), where further processing into miRNA duplexes by the protein Dicer occurs (Hutvagner et al., 2001). MiRNA duplexes associate with the RISC complex but only one strand, the mature miRNA, remains associated with it and is delivered to its target (Schwarz et al., 2003) (Khvorova et al., 2003). The fate of target mRNAs depends on the degree of complementarity with the miRNA. Commonly, the 5' 2-8 nt of the miRNA (called the seed sequence) is complementary to the target, and the remaining miRNA contains many mismatches. A low degree of complementarity results in translational repression,

whereas a high degree of complementarity results in cleavage of the mRNA followed by its eventual destruction (Kim, 2005). Other classes of small RNAs that function to repress mRNA have recently been described. (Li et al., 2009)

Figure 3. MiRNA biogenesis. Primary microRNA transcripts are transcribed by RNA Polymerase II. They may be transcribed independently or excised from the introns of protein-coding genes. Pri-miRNAs are then processed into precursor hairpin molecules by Drosha/DGCR8. Following export to the cytoplasm, pre-miRNAs are further processed to yield ~22 nt single-stranded mature miRNAs. The mature miRNAs, incorporated into the RNA-induced silencing complexes (RISCs) then bind to the 3'UTR of mRNAs, causing mRNA degradation or translational repression.



1.4 MICRORNAS AND CERVICAL CANCER

Distinct miRNA expression profiles have been linked to many cancers (Volinia et al., 2006). MiRNA coding sequences are often found near fragile sites in chromosomes, as well as near integration sites of HR-HPVs (Zhang et al., 2006). MiRNAs can serve as oncogenes or tumor suppressors. The first miRNA genes shown to be associated with cancer were miR-15 and miR-16. These two miRNAs are usually downregulated in chronic lymphocytic leukemia (CLL) (Calin et al., 2002). They have recently been shown to negatively regulate *Bcl-2*, an oncogene that is commonly overexpressed in many cancers (Cimmino et al., 2005). These miRNAs are transcribed as a cluster within the intron of a non-protein coding gene *Leu2* that is often deleted in CLL. The *let-7* family of miRNAs regulates the *Ras* oncogenes (Johnson et al., 2005). *Ras* genes contain activating mutations in approximately 15%-30% of cancers. The miRNAs controlling the expression of these oncogenes therefore serve as important regulators of cellular proliferation. Some cancers have shown downregulation of *let-7* family miRNAs resulting in reciprocal upregulation of *Ras*. This downregulation is most pronounced in lung cancers (Takamizawa et al., 2004), but is sporadically regulated in other tissues (Johnson et al., 2005).

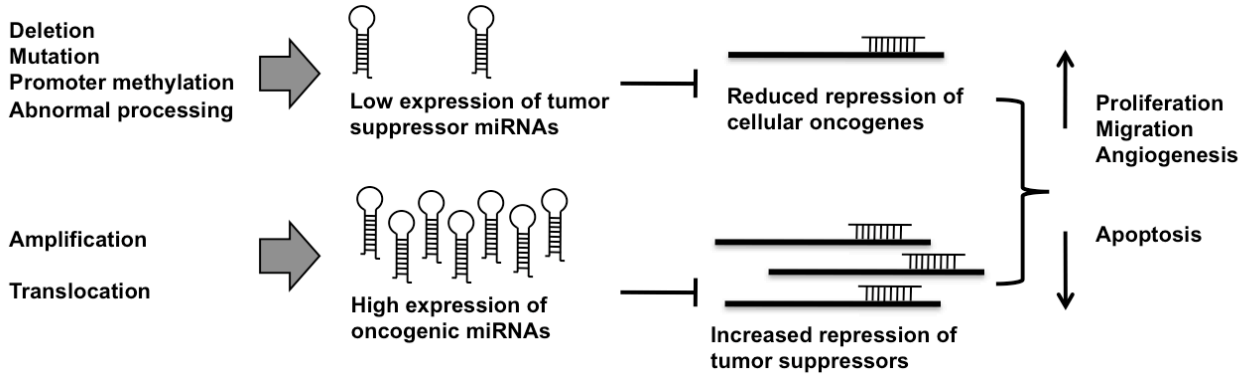
Oncogenes and tumor suppressors can also regulate the expression of miRNAs. C-myc regulates the miRNA cluster miR-17-92 that in turn targets E2F1 (O'Donnell et al., 2005). Upregulation of this cluster was demonstrated for B cell lymphomas (He et al., 2005). C-myc is often upregulated in cancers, and it serves as a transcription factor

for the miR-17-92 cluster (O'Donnell et al., 2005). Because C-myc directly targets E2F1, the C-myc-mediated repression of E2F1 via the miR-17-92 cluster serves to fine-tune its regulation (O'Donnell et al., 2005). Several studies have shown that p53, a major target of E6, is involved in the transcription and expression of many miRNAs (Xi et al., 2006). Methylation also plays a role in the regulation of miRNA expression (Saito et al., 2006) (Brueckner et al., 2007) (Lujambio et al., 2007). Changes in the expression of Dicer and other miRNA machinery proteins have been reported as well (Chiosea et al., 2006) (Chiosea et al., 2007). Many of the cell growth and proliferation pathways affected by HPV-16 contain probable targets or mediators of cellular miRNAs.

In cervical cancer, our studies showed that miR-218 is significantly underexpressed in HPV-16 positive cervical cancer cell lines and tissues (Martinez et al., 2008). Wang *et al.* found miR-15b, miR-16, miR-146a, and miR-155 to be most highly overexpressed in cervical cancer tissues and miR-126, miR-143, and miR-145 to be most underexpressed (Wang et al., 2008). They also found that miR-21, miR-181c, and miR-223 were overexpressed and miR-218 was underexpressed (Wang et al., 2008). Lui *et al*. analyzed the respective cloning frequency of miRNAs in cervical carcinoma cell lines versus normal cervical tissue (Lui et al., 2007). They did not amplify miR-218 in any of the HPV-positive cells. They further evaluated the expression levels of miR-21 and miR-143 and found significant overexpression of miR-21 and underexpression miR-143 in cervical cancer cell lines and tissues versus normal cervical tissue (Lui et al., 2007). Lee *et al.* analyzed 157 cellular miRNAs via the TaqMan® MicroRNA Human Early Panel Kit (Applied Biosystems) in 10 cervical cancer specimens and 10 normal cervical specimens (Lee et al., 2008). They found

overexpression of 68 miRNAs and underexpression of two miRNAs. They found that the 10 miRNAs that were most highly overexpressed were miR-199-s, miR-9, mir-199*, miR-199a, miR-199b, miR-145, miR-133a, miR-133b, miR-214, and miR-127. Only two miRNAs (miR-149 and miR-203) were found to be underexpressed (Lee et al., 2008).

Figure 4. MiRNAs and cancer. Loss of or low expression of miRNAs that target oncogenes may contribute to a cancer phenotype. High expression of miRNAs that target tumor suppressors may also lead to the cancer phenotype. (Garzon et al., 2006)



2.0 CHAPTER 2

HUMAN PAPILLOMAVIRUS TYPE 16 REDUCES THE EXPRESSION OF MICRORNA-218 IN CERVICAL CARCINOMA CELLS

Work described in this section was published in Oncogene (Oncogene. 2008, 27:2575-82) with authors Amy S. Gardiner, Ivan Martinez, Kathryn F. Board, Federico A. Monzon, Robert P. Edwards, and Saleem A. Khan. A.S. Gardiner and I. Martinez contributed equally to this work.

2.1 INTRODUCTION

High-risk human papillomaviruses (HPVs) such as types 16 and 18 are causally involved in cervical cancer (zur Hausen, 2002). HPVs are small double-stranded DNA viruses that contain two oncogenes E6 and E7 that are involved in cellular transformation. Most low grade cervical lesions contain HPV DNA in an episomal state, but in most cases of cervical carcinomas the HPV DNA is found integrated into the host chromosomes, increasing expression of E6 and E7 (Munger and Howley, 2002) (Hebner and Laimins, 2006). The E6 protein promotes ubiquitination and proteasomal degradation of the tumor suppressor protein p53 (Huibregtse et al., 1991) (Lechner et al., 1992) (Band et al., 1993) (Thomas et al., 1999) and PDZ domain-containing disc large protein (DLG) (Gardiol et al., 1999). The E7 protein binds to and inactivates the function of the pRB and related tumor suppressor proteins p107 and p130 (Munger and Howley, 2002). E7 also interacts with additional cellular proteins such as TBP, histone H1 kinase and cyclin E (Massimi et al., 1996) (Hebner and Laimins, 2006). In addition, E6/E7 expression promotes chromosomal instability, foreign DNA integration and other mutagenic events in the cell (Duensing et al., 2000) (Hebner and Laimins, 2006).

MicroRNAs (miRNAs) are small non-coding RNAs that may regulate thousands of mRNA targets, for reviews, see (Lewis et al., 2005) (Calin and Croce, 2006b). MiRNAs are transcribed in the nucleus and after processing they associate with the RISC complex and act as negative regulators of gene expression by binding to their complementary mRNA targets and either repressing translation or promoting mRNA

degradation (Kim, 2005). Recently, changes in the expression of miRNAs have been shown to be associated with a variety of human cancers (Calin and Croce, 2006a).

In this study, we demonstrate differential expression of several miRNAs in HPV-16 positive cervical cell lines and tissues, as well as in the HPV-18 positive cell line HeLa, compared to the normal cervical tissue and an HPV-negative cervical carcinoma cell line. We also demonstrate that miR-218 and the tumor suppressor gene *SLIT2* are specifically downregulated in several HPV-16 positive cervical cell lines and tissues, and this effect is mediated by the E6 oncogene of high-risk HPV-16. Finally, our studies show that *LAMB3* is a possible target of miR-218 at the transcriptional level.

2.2 MATERIALS AND METHODS

Cell lines

Cervical cancer cell lines CaSki, SiHa (HPV-16 positive), HeLa (HPV-18 positive), and C-33A (HPV-negative) and their growth conditions have been described (Meissner, 1999). Three clonal populations of the HPV-16 cervical cell line W12, 20863 (episomal HPV-16), 20861 and 201402 (integrated HPV-16), were obtained from the laboratories of Drs. Margaret Stanley and Paul Lambert (Medical Research Council, UK and University of Wisconsin, USA, respectively). The cell lines U2OS-Neo, U2OS-E6, U2OS-E7 (Duensing et al., 2000) were obtained from Dr. Stefan Duensing (University of Pittsburgh, USA). Normal oral keratinocytes (NOK) cell lines immortalized by hTERT expression and expressing the E6 gene of HPV-6 or HPV-16 (Piboonniyom et al., 2003)

were obtained from Drs. Stefan Duensing and Karl Munger (Harvard University).

Cervical tissues characteristics

Human cervical tissue samples were collected under an IRB approved protocol from patients with cervical preinvasive neoplasia undergoing leep excision or radical hysterectomy for invasive cervical cancer. Informed consent was obtained from all subjects. Parallel specimens of the same site were stained (Hemotoxylin and Eosin) to confirm the diagnosis. HPV-16 positivity was confirmed by RT-PCR. Total RNA from normal human cervix was obtained from a pool of 2 different donors (Stratagene). Prior to further RNA purification, these preparations contained limited levels of cellular DNA and were used to confirm the absence of HPV DNA. PCR amplification showed positive results for G3PDH and negative results for several HPV types using the degenerate MY09/MY11 primer pair (Manos et al., 1994). Furthermore, the preparations also did not give any PCR signals for the HPV-16 E7 and E2 genes. CaSki cells were used as a positive control and gave positive signals for the G3PDH gene as well as for the HPV L1 gene and HPV-16 E7 and E2 genes (data not shown).

MicroRNA microarray analysis

Total RNA was extracted using the Ultraspec™ RNA isolation system (BIOTECX, USA) according to the manufacturer's instructions. Total RNA from cell lines and normal cervix was used to isolate small RNAs (<200 nt) which includes miRNAs using the

RNeasy Mini Kit and the MinElute Cleanup Kit (Qiagen, USA). *MirVana* miRNA Bioarray (Ambion, USA) was used to analyze miRNA expression in cervical cell lines. The Bioarray consist of 662 probes (~22 nt long antisense oligonucleotides) that include known human miRNAs (328), theoretical human microarrays known as ambi-miRs (152) as well as unique miRNAs from mouse (114) and rat (46). Each array contains probes for all miRNAs in quadruplicate, and the signals obtained for each miRNA is represented as an average of these four values. The enriched small RNA fractions obtained from 25 μg of total RNA were fluorescently labeled and hybridized to the Bioarrays according to the manufacturer's instructions (Ambion, USA). Each experiment was done twice. The Bioarrays were scanned using GenePix 4000B Scanner and the median fluorescent intensity was obtained after subtracting the background using the GenePix Pro 6.0 software. To identify differential miRNA expression between samples, the median fluorescent intensities were log2 transformed and normalized using the median within the array and the global mean adjustment among arrays using the GEDA program (http://bioinformatics.upmc.edu/Help/GEDADescription.html). After transformation and normalization, we used the Significance Analysis of Microarray (SAM) program version 1.21 (www-stat.stanford.edu/~tibs/SAM/) to obtain the differential expression profiles.

MicroRNA Northern blot analysis

Enriched miRNA fractions obtained from 25 μg of total RNA were separated on 15% urea-containing polyacrylamide gels, transferred onto GeneScreen Plus

membranes and hybridized to 32P-labeled oligonucleotide probes complementary to various cellular miRNAs. We hybridized a probe complementary to the housekeeping splicing-related small U6 RNA for loading control. Hybridization was carried out overnight at 50C and the membranes were subjected to autoradiography at -80C.

MicroRNA and mRNA real-time quantitative RT-PCR analysis

DNase I-treated total RNA (10 ng) was subjected to qRT-PCR analysis using the TaqMan® miRNA Reverse Transcription Kit and miRNA Assays (Applied Biosystems, USA), and the Real-Time thermocycler iQ5 (BioRad, USA). The small nucleolar RNU43 was used as the housekeeping small RNA reference gene. For qRT-PCR analysis of *SLIT2* and *LAMB3* mRNAs, the following primers were used: (*SLIT2*, forward 5'-CTGTGAATGCAGCAGTGGAT-3' and reverse 5'-TTGTTTGGCAAGCAGCATAG-3' (116-bp product); *LAMB3*, forward 5'- GGGAGACCATGGAGATGATG-3'and reverse 5'-ACACGCTTCTCCAGTCCTGT-3' (112-bp product). Also, 500 ng of total RNA from the cervical samples was amplified using the one step QuantiTect SYBR Green RTPCR Master Mix (Qiagen, USA). The primer sequences for the control housekeeping glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH) have been described previously (Martinez *et al*., 2007). All reactions were done in triplicate and relative expression of RNAs was calculated using the 2 delta CT method (Livak and Schmittgen, 2001).

HPV-16 E6 and E7 siRNA and transfection assays

Double-stranded small interfering RNAs (siRNAs) against HPV-16 E6 (siRNA 209, 5'-UCCAUAUGCUGUAUGUGAUTT-3'; complementary to HPV-16 positions 277 to 298) and E7 (E7 siRNA, 5'-CCAUCUAUUUCAUCCUCCUTT-3', complementary to HPV-16 positions 662 to 682) (Jiang and Milner, 2002) (Tang et al., 2006) were obtained from Dharmacon, USA. BLOCK-iT fluorescent double-stranded oligo (with no human homologous sequence) was used as a negative control as well as to measure the transfection efficiency (Invitrogen, USA). MiR-218 was expressed in cell lines by transfecting with a pre-miR-218 precursor molecule (Ambion, USA). Cell lines were seeded (1.5 X 10^5) into 6-well plates, and after 24 hr, transfected (125 nM per well of HPV siRNAs or 100 nM of miR-218 precursor molecule) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells were harvested after 72 hr, and RNA and protein extractions were performed.

Western blot analysis

Twenty micrograms of total cellular proteins were separated on 7.5% SDS polyacrylamide gels, transferred to Immobilon-P PVDF membranes (Millipore), and incubated with primary murine monoclonal antibody (Calaluce et al., 2004) against the β3 chain of laminin-5 (Kalinin B1; BD Biosciences, USA). The membranes were subsequently incubated with a 1:5,000 dilution of the secondary anti-mouse horseradish peroxidase antibody (Amersham Biosciences, USA). Specific proteins were detected using chemiluminescence with ECL Plus Western Blotting Detection Reagents

(Amersham Biosciences, USA). Murine monoclonal antibody against G3PDH (Chemicon) was used to demonstrate equal loading.

2.3 RESULTS

Differential expression of microRNAs in cervical cell lines compared to the normal cervix and the HPV-negative cell line C-33A

MiRNA microarray analysis showed that approximately 220 known human miRNAs out of 328 represented on the array were expressed in the normal cervix (Supplementary Table 1). The miRNAs that were most highly expressed in the cervix were miR-145, miR-26a, miR-99a, let-7a, miR-143, let-7b, let-7c, miR-125b, miR-126, and miR-195 in that order. We investigated the miRNA expression profile in normal cervical tissue and cervical carcinoma cell lines SiHa and CaSki containing integrated HPV-16 DNA. We also used two clonal derivatives, 20861 and 201402, of the W12 cell line derived from a low-grade CIN I lesion (Stanley et al., 1989), which contain integrated HPV-16 DNA (Alazawi et al., 2002). SAM analysis of the array data showed that 24 miRNAs, including miR-126, miR-143, miR-145 and miR-195 (four of the ten most highly expressed miRNAs in the normal cervix) were underexpressed in all the integrated HPV-16 cervical cell lines compared to the normal cervix (Table 1). Only three miRNAs, miR-182, miR-183 and miR-210, were found to be overexpressed in the integrated HPV-16 cell lines (Table 1). The array data for individual cell lines compared

Table 1. MiRNAs differentially expressed in HPV-16 positive cell lines compared to the normal cervix

MiRNA	HPV-16 integrated Fold[a]
Overexpressed	
hsa_miR_210	7.3
hsa_miR_182	6.4
hsa_miR_183	5.1
Underexpressed	
hsa_miR_126	-14.5
hsa_miR_145	-14.1
hsa_miR_451	-11.3
ambi_miR_7029	-9.5
hsa_miR_195	-8.3
hsa_miR_143	-8
hsa_miR_199b	-7.9
hsa_miR_133a	-7.6
hsa_miR_368	-7.6
hsa_miR_1	-7.2
hsa_miR_495	-6.3
hsa_miR_497	-6.2
hsa_miR_133b	-6
hsa_miR_223	-5.4
hsa_miR_146a	-5.1
hsa_miR_218	-4.8
hsa_miR_126_AS	-4.7
hsa_miR_150	-4.4
hsa_miR_376a	-4.2
hsa_miR_214	-4.1
hsa_miR_487b	-4.1
hsa_miR_10b	-3.9
ambi_miR_5021	-3.6
ambi_miR_7070	-3.5

[a]Mean fold-changes in HPV-16 integrated cell lines CaSki, SiHa, 20861 and 201402. The *q* value of all miRNAs was 0.

Table 2. MiRNAs differentially expressed in HPV-16 positive cell lines compared to the HPV-negative cell line C-33A

MiRNA	HPV-16 integrated Fold[a]
Overexpressed	
hsa_miR_200c	27.9
hsa_miR_203	23.4
hsa_miR_193b	21.2
hsa_miR_34a	10.4
hsa_miR_31	8.4
hsa_miR_210	5.7
hsa_miR_27a	5.4
hsa_miR_503	5.4
hsa_miR_27b	4.9
Underexpressed	
hsa_miR_218	-7.4

[a]Mean fold-changes in HPV-16 integrated cell lines CaSki, SiHa, 20861 and 201402. The *q* value of all miRNAs was 0.

to the normal cervix is presented in Supplementary Table 2. In the 20863 cell line (a clonal derivative of the W12 cell line) containing episomal HPV-16 DNA, six miRNAs were underexpressed compared to the normal cervix (Supplementary Table 2). A direct comparison of the miRNA expression profiles of integrated vs. episomal HPV-16 cell lines did not reveal any significant differences (data not shown). This, at least in part, may result from the use of only one cell line containing episomal HPV-16 DNA, as well as the use of a 2-fold cut-off value and a q value of zero in our statistical analysis.

MicroRNA expression profile of the HPV-18 containing HeLa cell line showed that 14 miRNAs were underexpressed in HeLa cells compared to the normal cervix (Supplementary Table 3). Eight of these miRNAs (miR-1, miR-133b, miR-143, miR-145, miR-214, miR-368, miR-451 and miR-7029) were also found to be underexpressed in cell lines containing integrated HPV-16 DNA (Table 1). Thirteen miRNAs were found to be overexpressed in the HeLa cell line compared to the normal cervix (Supplementary Table 3). Of these, two miRNAs (miR-182 and miR-183) were also overexpressed in the HPV-16 integrated cell lines (Table 1).

Nine miRNAs in cell lines containing integrated HPV-16 DNA were found to be expressed at much higher levels compared to the HPV-negative cervical carcinoma cell line C-33A cells (Table 2). Interestingly, miR-218 was the only miRNA that was underexpressed in the cell lines containing integrated HPV-16 DNA as compared to both the normal cervix and C-33A (Tables 1 and 2). This suggested that miR-218 may be specifically affected in the presence of HPV-16. The array data for individual cell lines compared to C-33A is presented in Supplementary Table 4.

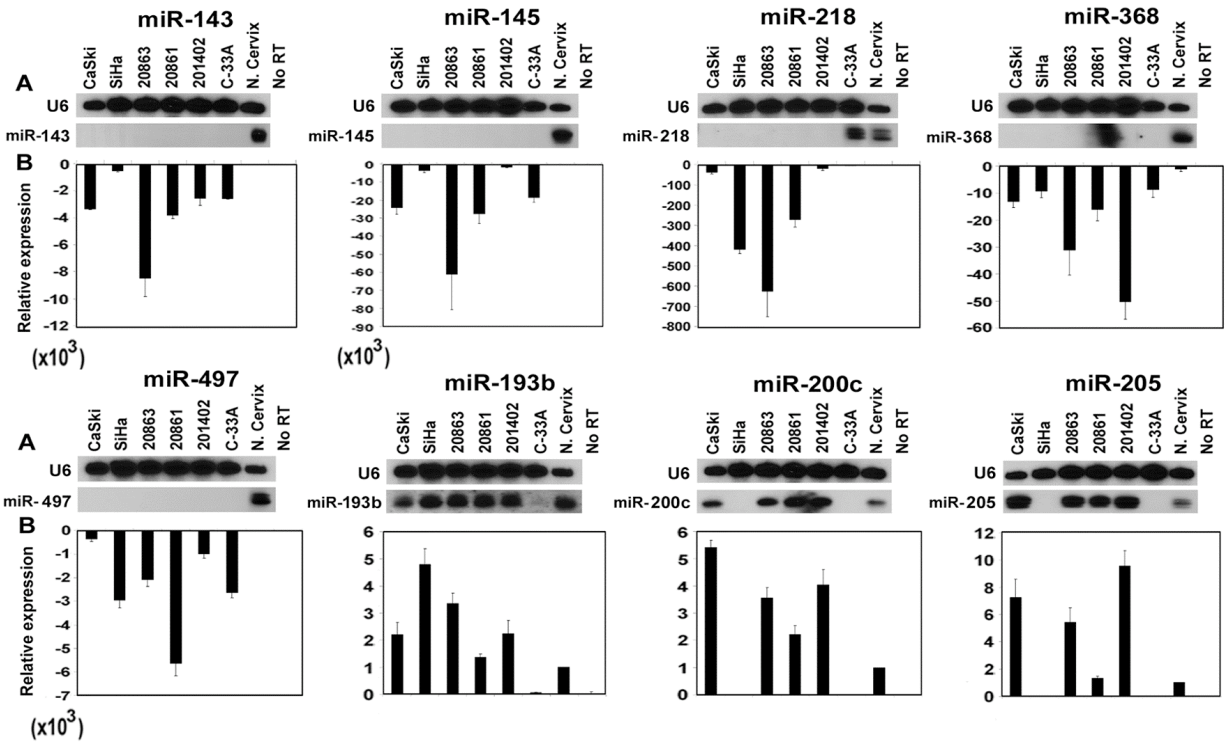
The HPV-18 positive HeLa cell line showed overexpression of six miRNAs

compared to the HPV-negative cell line C-33A (Supplementary Table 5). Of these, three miRNAs (miR-31, miR-34a and miR-193b) were also overexpressed in integrated HPV-16 cell lines compared to C-33A (Table 2). Finally, the array data for the HPV-negative cell line C-33A showed that 4 miRNAs (miR-143, miR-145, miR-200c and miR-203) were underexpressed as compared to the normal cervix (Supplementary Table 6). In addition, qRT-PCR and Northern blot analyses showed that miR-193b, miR-205 and miR-497 were also downregulated in the C-33A cell line compared to the normal cervix (see below).

Validation of the miRNA microarray expression data by quantitative real-time RT-PCR and Northern blot analyses

The miRNA microarray results were validated by qRT-PCR and Northern blot analyses of 8 representative miRNAs representing those whose expression was either most affected, was known to be affected in other types of cancers, or one that appeared to be HPV-specific (miR-218) (Figure 5A, B). MicroRNAs 143, 145, 218, 368 and 497 which were found to be downregulated in HPV-16 positive cell lines in array data were also found to be underexpressed based on qRT-PCR and Northern blot analyses (Figure 5A, B), although there were differences in the relative levels of individual miRNAs in various cell lines. The fold-changes observed by qRT-PCR and Northern blot analyses were much greater than those obtained with the microarrays. This was observed consistently and suggests that the results of qRT-PCR and Northern blot analyses are more robust for quantifying differential expression of miRNAs. The qRT-

Figure 5. Confirmation of miRNA microarray expression data in various cervical cell lines and normal cervical tissue. (A) Northern blot analysis. The housekeeping splicing-related small U6 RNA was used as a loading control. (B) Real-time qRT-PCR analysis. RNU43 served as the endogenous control for miRNAs.



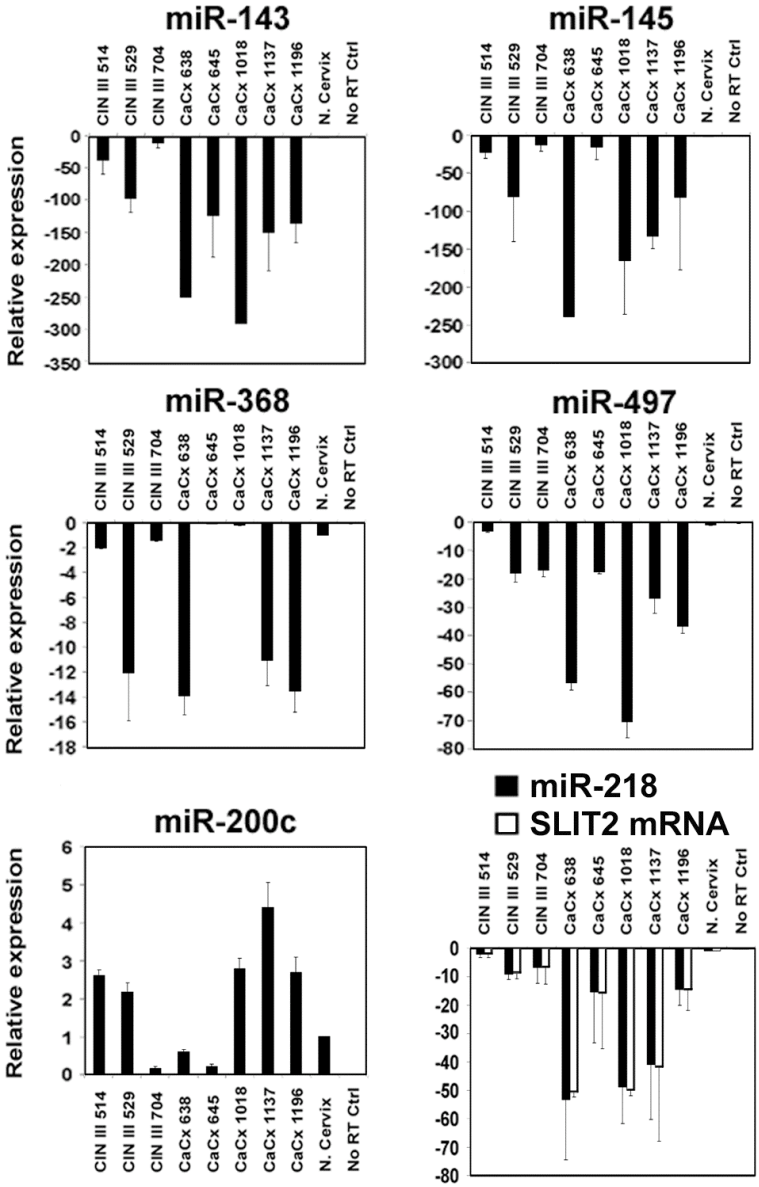
PCR and Northern blot analyses revealed that miR-200c and 205 were upregulated in all the HPV-positive cell lines except SiHa compared to the normal cervix (Figure 5A, B). These two miRNAs were not detectable in SiHa and the HPV-negative cell line C-33A (Figure 5A, B). The “band” seen in the Northern blot for miR-368 in the 20861 sample is a gel artifact and does not represent a miRNA signal.

The array data obtained with the HPV-18 positive HeLa cell line were also validated by Northern blot and qRT-PCR analyses of three miRNAs, miR-143, miR-145, and miR-218 all of which were found to be downregulated by the various techniques (Supplementary Figure 1).

MicroRNA 218 is also underexpressed in HPV-16 positive cervical lesions and cervical cancer tissues

We also analyzed the expression profile of six representative miRNAs (all of which were also used for the validation of the array data for the cell lines) in three HPV-16 positive cervical intraepithelial neoplasia grade III (CIN III) tissues and five HPV-16 positive cervical cancer tissues (CaCx) by qRT-PCR analysis. Due to the limited amount of RNA available from the tissues, miRNA microarray analysis could not be done. The expression pattern of the above miRNAs in the tissues (Figure 6) was generally consistent with that of the HPV-positive cell lines. Importantly, miR-218 was found to be underexpressed (>2-fold reduction) in all the CIN III and CaCx samples compared to the normal cervix (Figure 6). On average, the CIN III samples showed a more limited

Figure 6. Expression of miRNAs and the *SLIT2* gene in cervical tissues. qRT-PCR analysis of three cervical intraepithelial neoplasias type III (CIN III) and five cervical carcinomas (CaCx). The normal cervix sample was obtained from Stratagene. G3PDH served as the endogenous control for *SLIT2*.

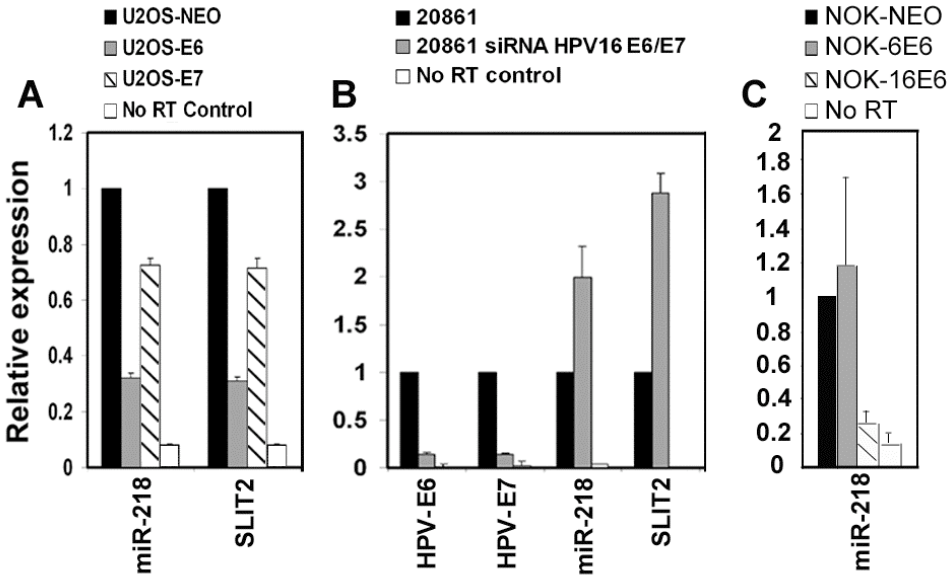


underexpression of miR-218 compared to the CaCx tissues. Since miR-218 is encoded by an intron of the *SLIT2* tumor suppressor gene (Griffiths-Jones *et al*., 2006), we tested whether their expression is correlated. Previous analysis of global gene expression preformed in our lab via Human Genome U133A 2.0 Arrays (Affymetrix) also showed that *SLIT2* was underexpressed in the HPV-positive cell lines. The qRT-PCR results showed that *SLIT2* expression paralleled that of miR-218, and both of these were underexpressed in the CIN III and CaCx tissues (Figure 6). MiRNAs 143, 145 and 497 that were underexpressed in the HPV-16 positive cell lines were also underexpressed in the HPV-positive tissues compared to the normal cervical tissue (Figure 6), although the relative levels of various miRNAs varied between the individual samples. In the case of miR-368, 5 out of 8 cervical cancer and CINIII lesions showed downregulation as compared to the normal cervix (Figure 6). Overall, the results obtained with the tissues provide further validation of the data obtained with the cervical cell lines.

HPV-16 E6 oncogene downregulates miR-218

To test whether E6 and/or E7 expression is directly correlated with reduced expression of miR-218, we utilized the osteosarcoma cell line U2OS either expressing the HPV-16 E6 or E7 gene, or the control neomycin resistance gene. The qRT-PCR results showed that both miR-218 and *SLIT2* were underexpressed in the U2OS-E6 cell line compared to U2OS-E7 and the control U2OS-Neo cell line (Figure 7A). In another approach, the 20861 cell line containing integrated HPV-16 was transfected with HPV-16 E6/E7 siRNAs. Since E6 and E7 are derived from alternative splicing of the same

Figure 7. HPV-16 E6 oncogene reduces the expression of miR-218. (A) qRT-PCR analysis of miR-218 and SLIT2 in U2OS-NEO, U2OS-16E6, and U2OS-16E7. (B) Expression of HPV-16 E6 and E7, miR-218 and *SLIT2* in the 20861 cell line with or without RNAi against HPV-16 E6/E7. (C) qRT-PCR analysis of miR-218 in NOK-NEO, NOK cell line expressing the E6 gene of either the low-risk HPV-6 (NOK-6E6) or high-risk HPV-16 (NOK-16E6). RNU43 served as the endogenous control for miRNAs, while G3PDH served as the endogenous control for E6, E7 and *SLIT2*.

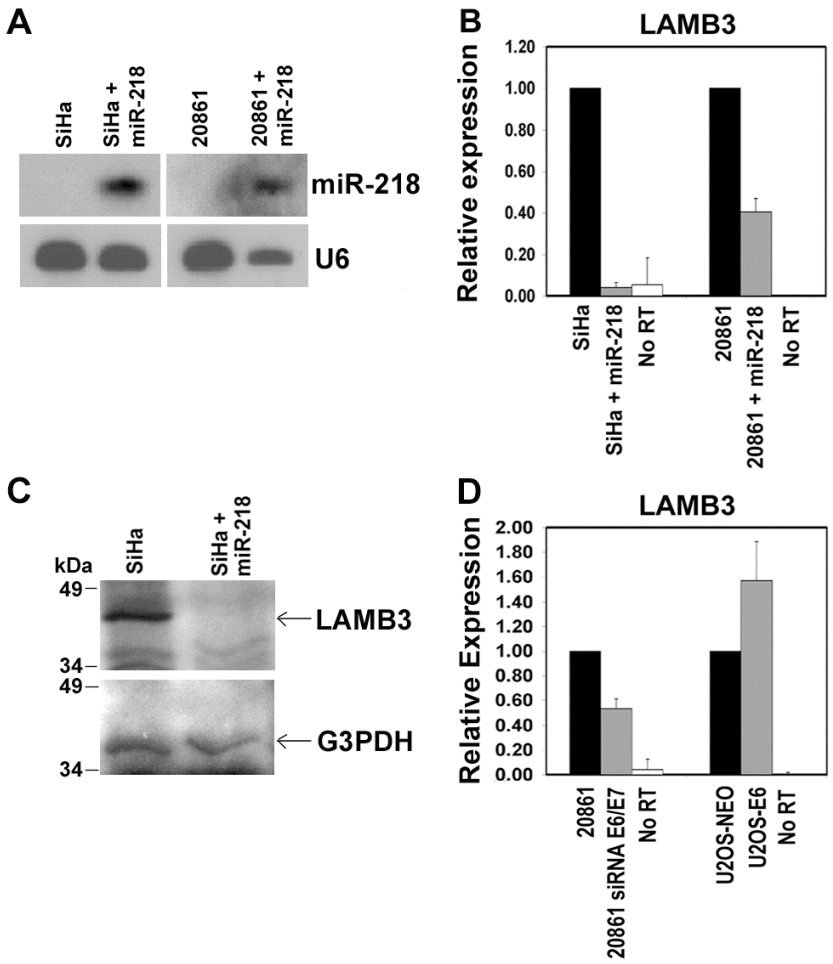


RNA, a specific siRNA for E6 alone could not be used. The E6/E7 siRNAs reduced expression of these genes while increasing the expression of both miR-218 and the *SLIT2* gene in 20861 cells (Figure 7B). These results indicate that the HPV-16 E6 gene is involved in the downregulation of miR-218 and the *SLIT2* gene in HPV-16 positive cell lines. Since a U2OS derivative expressing the E6 gene of a low-risk HPV is not available, we utilized normal oral keratinocytes (NOK) expressing the HPV-6 E6 gene to study whether the E6 gene of a low-risk HPV also affects miR-218 expression. The qRT-PCR analysis showed that NOK-16E6 cells had reduced expression of miR-218 compared to both NOK-NEO and NOK-6E6 (Figure 7C). These results suggest that the E6 gene of the high-risk HPV-16, but not the low-risk HPV-6, reduces miR-218 expression.

Laminin 5 β3 is a transcriptional target of miR-218

To identify possible miR-218 targets, we compared computationally predicted targets in the miRBase Registry (Griffiths-Jones et al., 2006) with our gene expression data (manuscript in preparation), and then used the program rna22 (http://cbcsrv.watson.ibm.com/rna22.html). This analysis revealed six possible targets of miR-218: emopamil binding protein (EBP), mitochondrial ribosomal protein S27 (MRPS27), nucleoporin 93kDa (NUP93), ephrin-A1 (EFNA1), laminin 5 β3 (LAMB3) and muscleblind-like 2 (MBNL2). The expression of these genes was analyzed by qRTPCR in SiHa and 20861 cell lines transfected with an artificial miR-218 precursor molecule. After confirming the expression of mature miR-218 (Figure 8A), we found that

Figure 8. Expression of *LAMB3* is reduced in the presence of miR-218. (A) Northern blot analysis of miR-218 after transfection of a precursor of miR-218 in SiHa and 20861 cell lines. U6 RNA was used as a loading control. (B) qRT-PCR analysis of *LAMB3* in SiHa and 20861 cell lines transfected with a pre-miR-218. (C) Western blot analysis of LAMB3 protein in SiHa cells transfected with pre-miR-218. Location of the 40-kDa LAMB3 protein is indicated. G3PDH was used as a control. (D) Expression of *LAMB3* mRNA in the 20861 cell line with or without RNAi against HPV-16 E6/E7, and in the U2OS-NEO and U2OS-16E6 cell lines. G3PDH served as the endogenous control for *LAMB3*.



only the *LAMB3* transcript was significantly underexpressed in miR-218 expressing cells (Figure 8B and data not shown). Furthermore, Western blot analysis showed that miR-218 expression also greatly reduced the levels of the LAMB3 protein in SiHa cells (Figure 8C). We also found that *LAMB3* was underexpressed in the 20861 cell line in the presence of the E6/7 siRNAs compared to a control oligo (Figure 8D). Furthermore, U2OS-16E6 cells showed an increase in the levels of *LAMB3* mRNA as compared to the U2OS-NEO cells (Figure 8D). Taken together, these results demonstrate that miR-218 reduces *LAMB3* expression at the transcriptional level.

2.4 DISCUSSION

The microarray data showed that 24 miRNAs were underexpressed and 3 overexpressed in integrated HPV-16 cell lines compared to the normal cervix (Table 1). We also validated these data by Northern blot and qRT-PCR analyses of five miRNAs that were underexpressed (miR-143, miR-145, miR-218, miR-368 and miR-497) and three that were overexpressed (miR-193b, miR-200c and miR-205) in the HPV-16 positive cell lines compared to the normal cervix or the HPV-negative cervical carcinoma cell line C-33A (Figure 5). The probable targets of the above eight miRNAs are shown in Supplementary Table 7. The observed differences in the levels of differentially expressed miRNAs were generally much greater in qRT-PCR and Northern blot studies as compared to the array data. Thus, the array data likely underestimate the fold-changes in the levels of differentially expressed miRNAs. Our studies identified 10 underexpressed miRNAs in integrated HPV-16 cell lines that are not known to be

altered in any cancers. These included 7 known human miRNAs, miR-1, miR-126-AS, miR-133a, miR-376a, miR-451, miR-487b, miR-495, and 3 predicted miRNAs, ambi-miR-5021, ambi-miR- 7029, and ambi-miR-7070. MiR-143 and miR-145, which are underexpressed in colon and breast cancers (Calin and Croce, 2006a), were also underexpressed in all cervical cell lines including C-33A (Tables 1 and 3), suggesting that they may be important in cervical carcinogenesis independent of HPV infection. MiR-368, miR-497 and miR-193b which are underexpressed in integrated HPV-16 cell lines are known to be underexpressed in colon cancer cell lines (Bandres et al., 2006), papillary thyroid carcinomas (Calin and Croce, 2006a), and colon cancer (Cummins et al., 2006), respectively. Interestingly, miR-218 was the only miRNA found to be underexpressed in integrated HPV-16 cell lines compared to both the normal cervix and C-33A cells (Tables 1 and 2, and Figure 5). Northern blot and qRT-PCR analysis showed that miR-218 is also underexpressed in HeLa cells containing integrated HPV-18 DNA and the 20863 cell line containing episomal HPV-16 DNA (Figure 5, and Supplementary Figure 1). These results suggest that miR-218 may be a specific cellular target of high-risk HPVs. MiR-218 is known to be underexpressed in several cancers and the DNA encoding miR-218 is also deleted in ovarian, breast, and melanoma cancers (Calin and Croce, 2006a) (Zhang et al., 2006). Of the miRNAs overexpressed in integrated HPV-16 cell lines, miR-210 is overexpressed in many epithelial cancers (Calin and Croce, 2006a), while miR-182 and miR-183 are overexpressed in colon cancer cell lines (Bandres et al., 2006). We also found that 6 miRNAs have reduced expression in the episomal HPV-16 cell line 20863 compared to the normal cervix (Supplementary Table 2). These miRNAs may represent early targets of HPV-16

infection. A comparison of the miRNA expression profile of HPV-16 and HPV-18 positive cell lines with that of the normal cervix showed that while some miRNAs are similarly affected in the HPV-16 and HPV-18 positive cells, others are not. This may indicate the presence of both common as well as unique pathways that are altered in cells containing HPV-16 and HPV-18 DNA.

Analysis of 6 representative miRNAs, miR-143, miR-145, miR-200c, miR-218, miR-368 and miR-497 (whose expression levels in various cell lines were also analyzed by a variety of methods) in CIN III and CaCx tissues by qRT-PCR showed expression patterns that were generally similar to those of the HPV-positive cell lines (Figures 5 and 6). Importantly, miR-218 was also underexpressed (>2-fold difference) in all the CIN III and CaCx tissues (Figure 6). These results suggest that miR-218 underexpression is likely linked to the process of HPV-associated carcinogenesis *in vivo*. The parallel expression of miR-218 and the *SLIT2* gene (which encodes miR-218) in all the tumor tissues (Figure 6) suggests that they may be coordinately regulated. Previous studies have shown that the *SLIT2* tumor suppressor gene is frequently inactivated in lung and breast cancers (which have reduced miR-218 levels) (Dallol et al., 2002). Whether regulation of *SLIT2* plays a role in the pathogenesis of HPV-associated malignancies is currently unknown.

Our results showed that both miR-218 and *SLIT2* were underexpressed in the U2OS-16E6 cell line (Figure 7A). Similarly, miR-218 was also underexpressed in NOK-16E6 but not in the NOK-6E6 cell line (Figure 7C). These results suggest that E6 of the high-risk HPV-16, but not the low-risk HPV-6, may contribute to the downregulation of miR-218. This possibility is also supported by our observations that expression of E6/E7

siRNAs in 20861 cells increases miR-218 and *SLIT2* mRNA levels (Figure 7B). Interestingly, *SLIT2* is a possible target of miR-200c (overexpressed in the HPV-positive cell lines and tissues) (Supplementary Table 7). One study has shown that the absence of the p53 gene increases miR-200c expression (Xi et al., 2006). Thus, it is possible that E6-dependent degradation of the p53 protein results in miR-200c overexpression which in turn reduces the levels of miR-218 and *SLIT2* mRNA.

Our studies showed that introduction of miR-218 into SiHa and 20861 cell lines reduced the levels of Laminin 5 β3 (*LAMB3*) mRNA as well as the protein (Figure 8A, B, C). This strongly supports implies that miR-218 can downregulate *LAMB3* expression independent of *SLIT2*. Furthermore, introduction of E6/E7 siRNA into the 20861 cell line also reduced *LAMB3* expression (Figure 8D). Also, E6 expression in U2OS cells increased *LAMB3* expression (Figure 8D) presumably by reducing miR-218 expression. Taken together, the above studies suggest that miR-218 may regulate *LAMB3* expression at the transcriptional level. LAMB3 protein is part of the polymeric cell surface receptor laminin 5 that is expressed in the basal lamina of the epithelium and is overexpressed in cervical cancers (Skyldberg et al., 1999) (Kohlberger et al., 2003). LAMB3 increases cell migration and tumorigenicity in SCID mice, and in collaboration with its ligand α6β4-integrin promotes tumorigenesis in human keratinocytes (Dajee et al., 2003) (Calaluce et al., 2004). A recent study suggests that secreted laminin 5 can be used by HPV as a transient receptor to aid the virus in the infection of basal cells that express α6β4-integrin (Culp et al., 2006). Thus, downregulation of miR-218 by E6 and the consequent overexpression of *LAMB3* may promote viral infection of the surrounding tissue and contribute to eventual tumorigenesis.

3.0 CHAPTER 3

MICRORNA ANALYSIS IN HUMAN PAPILLOMAVIRUS (HPV)-ASSOCIATED CERVICAL NEOPLASIA AND CANCER

Work described in this section has been submitted for publication. The authors are Amy S. Gardiner, William C. McBee, Jr., Robert P. Edwards, Jamie L. Lesnock, Rohit Bhargava, R. Marshall Austin, Richard S. Guido, and Saleem A. Khan. Amy S. Gardiner and William C. McBee Jr. contributed equally to this work.

3.1 INTRODUCTION

Worldwide, cervical cancer is the most common gynecologic malignancy. The estimated incidence in 2002 was 493,243 new cases and an associated 273,505 related deaths, making it the third most deadly cancer, behind breast and lung cancers (Parkin et al., 2005). Cervical cancer is a major health concern in the United States as well. In 2008 there were an estimated 11,070 new cases and an associated 3,870 deaths, accounting for approximately 1% of cancer deaths in women. Developed countries such as the United States have seen a significant decrease in the incidence of invasive cervical cancer during the past 50 years. This is largely due to the widespread use of effective screening techniques. However, the incidence of invasive cervical cancer still remains disproportionately higher in minorities and others with limited access to healthcare.

Due in large part to increased screening for cervical cancer, the incidence of patients diagnosed with cervical dysplasia has increased exponentially over the past 50 years. Currently, it is estimated that between 250,000 and 1,000,000 women in the United States will be diagnosed with cervical intraepithelial neoplasia (CIN) annually. The stepwise progression of mild to moderate to severe dysplasia, and eventually to invasive cervical cancer has been well documented. The management of patients with cervical dysplasia should be focused on the prevention of invasive cervical cancer. There is a huge expenditure required to diagnose, follow, and treat patients with cervical dysplasia, in order to prevent the development of invasive cancer.

Human papillomaviruses (HPVs) are the causative agents in essentially all cervical dysplasia and invasive cervical cancer (Walboomers et al., 1999). There are more than 100 documented HPV types, and approximately forty have the propensity to cause malignant changes, in varying degrees, in the anogenital epithelium (de Villiers et al., 2004). HPV infection is extremely common in sexually active individuals, with some reported lifetime incidence rates as high as 80% in women by the age of 50 (Ho et al., 1998). While the overall lifetime incidence of HPV infection in women is high, the virus is usually transient and the majority of infections are cleared in less than a year (Ho et al., 1998). Nevertheless, some patients are found to have cervical dysplasia that is often persistent and often more virulent. The ability to identify which cervical HPV infections will become cancers would markedly reduce our resource utilization currently required to prevent cervix cancer. Such a reduction would presumably make cervical cancer prevention more affordable to the third world where it remains a significant problem. HPVs are small, circular, non-enveloped, double stranded DNA viruses, about 8000 base pairs in size (zur Hausen, 2002). Early in HPV infection, the non-structural genes E1, E2, E4, E5, E6, and E7 are expressed. Later in the viral life cycle, the late structural genes L1 and L2 are expressed. This coincides with a burst of viral replication and transcription; virions are shed which infect surrounding tissue (Lee and Laimins, 2004). The E6 and E7 oncogenes work synergistically to deregulate cell cycle controls through a variety of mechanisms. The E6 oncogene promotes ubiquitination and proteasomal degradation of the tumor suppressor protein p53 and also deregulates the cell cycle (Scheffner et al., 1990) (Thomas et al., 1999) (Werness et al., 1990). The E7 protein binds to and inactivates the function of pRB and the related tumor suppressor proteins

p107 and p130 (Davies et al., 1993). Expression of E6 and E7 promotes chromosomal instability, foreign DNA integration, and other mutagenic events in the cell (Duensing et al., 2000) (Pett et al., 2004). This chromosomal instability is a hallmark of cancer.

MicroRNAs (miRNA) are small, non-coding RNAs (approximately 22 nucleotides in length) that regulate gene expression (Ambros, 2003). MicroRNAs function by binding to the 3' UTRs of their target messenger RNA (mRNA), whereby they induce mRNA degradation or repression of translation (Farh et al., 2005). The functions of miRNAs are still largely unknown but they appear to be integral to modulation of gene expression and cell behavior. Recent studies have suggested that they may play a role in the development of a variety of cancers (Calin and Croce, 2006a). Differentially expressed miRNAs may serve as early biomarkers for the progression of cervical dysplasia. Furthermore, the development of a biomarker panel may be useful for determining which patients with cervical dysplasia are likely to have disease progression and may also be a useful prognostic indicator in patients with invasive cancer. We compared the miRNA expression profiles in cervical cancer, dysplasia and normal cervix, and we report the identification of several miRNAs whose dysregulation is associated with the progression of HPV induced disease from mild dysplasia to invasive cancer.

3.2 MATERIALS AND METHODS

Cervical Tissue Collection and Characterization

After IRB approval had been obtained, prospective collection of cervical tissue was performed from various grades of neoplasia. Patients with preinvasive and grossly invasive cervical lesions undergoing excision were recruited for adjacent biopsies of the cervical lesion. One biopsy was snap frozen for our analysis and the other was sent to pathology for tissue diagnosis. Patients with microinvasive disease or prior radiation were excluded.

Cervical dysplasia specimens were collected from patients at the time of colposcopy and excision using a similar paradigm of adjacent biopsy techniques. After the LEEP excision was performed, a small sample from the area of suspected dysplasia was removed for miRNA analysis. The LEEP specimen was then sent for pathologic review. In addition to the standard pathologic review, the gynecologic pathologist evaluated the cervical tissue directly adjacent to the tissue that was removed for analysis. We used this analysis to grade the level of dysplasia in our sample. We only analyzed CIN 2 and 3 lesions. Any tissue collected with mild, or no dysplasia, was not processed.

Normal cervical tissue was also collected from controls, age-matched to the cervical cancer patients. This tissue was obtained from the Magee-Womens tissue bank (from a separate IRB) for patients with normal cervical cytology who had undergone a hysterectomy for a benign indication. Normal cervical samples were obtained from the transformation zone, to correspond to that taken from the dysplasia and cancer patients. All cervical tissue was directly evaluated by a gynecologic pathologist.

Once tissue was collected, it was snap frozen and stored at -80°C for later use. Next, a piece of the tissue was removed for DNA isolation and another for RNA

isolation. After DNA isolation, tissue samples were confirmed to be either HPV-positive or HPV-negative by PCR analysis using the MY09/MY11 primer set for amplification of the highly conserved HPV L1 structural gene (Manos et al., 1994). Amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a control, using the forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′. HPV-positive samples were then tested for the presence of HPV type 16 or 18 by PCR analysis of their respective E6 oncogene. The primers used were 5′-ATGCACCAAAAGAGAACTGC -3' (forward) and 5'-TTACAGCTGGGTTTCTCTAC-3' (reverse) for HPV-16 E6 and 5'-CGGCGACCCTACAAGCTACCTG-3' (forward) and 5'-CTGCGTCGTTGGAGTCGTTCC-3' (reverse) for HPV-18 E6. The majority of the tissues were HPV-16 positive, and we proceeded with the analysis of only such samples (dysplasia or cancer). The HPV-negative normal cervical tissues were also confirmed to have completely benign pathology.

Total RNA was isolated from all the cervical tissues using the Ultraspec™ RNA Isolation System (Biotecx, Houston, TX, USA) and analyzed by agarose gel electrophoresis. The HPV E6 and E7 oncogenes have been shown to be required for cellular transformation and directly responsible for cervical disease progression. Therefore, DNase-I-treated total RNA (1 μg) from HPV-16 positive tissues was subjected to RT-PCR analysis for the expression of the E6 and E7 oncogenes using the forward primer 5′-GTAACCTTTTGTTGCAAGTGTGACT-3′ and the reverse primer 5′-GATTATGGTTTCTGAGAACAGATGG-3′, and the Advantage® Clontech RT-for-PCR Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions.

MicroRNA Real-time Quantitative RT-PCR Analysis

We carried out miRNA expression analysis using the TaqMan® MicroRNA Reverse Transcription Kit and Megaplex™ RT Primers, followed by TaqMan® Human MicroRNA Arrays V2.0 from Applied Biosystems, which are designed for the sensitive amplification and profiling of 667 unique human miRNAs. The arrays also contain primers for three endogenous small RNAs U6, RNU44, and RNU48, which serve as positive controls, as well as primers for an *A. thaliana* miRNA, which serves as a negative control. This approach is based on real-time quantitative PCR and we used 1.5 µg of total RNA per sample for miRNA expression analysis. Amplification of all miRNAs was carried out according to the manufacturer's instructions and the samples were analyzed on the 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative quantification of miRNAs was performed using the 2 delta Ct method, where change in expression of a gene in an experimental sample is quantified relative to the same gene in a reference sample. We used two-fold as the cut-off ratio for identification of differentially expressed miRNAs. Data analysis was performed first by comparing cervical cancer tissue to normal cervical tissue for all overexpressed and underexpressed miRNAs, using a statistical significance level of $p=0.05$.

MiRNAs found to be overexpressed or underexpressed in the cancer or dysplasia samples as compared to the normal cervical tissue were further analyzed by individual quantitative RT-PCR (qRT-PCR) analysis using the TaqMan® MicroRNA Reverse Transcription Kit and individual TaqMan® MicroRNA Assays (Applied

Biosystems) according to the manufacturer's instructions. The RNA used in these experiments was normalized to the small nucleolar (Winn et al.) RNU43 levels and relative expression levels of miRNAs were calculated using the 2 delta CT method.

3.3 RESULTS

Patients were identified for tissue collection from March 2008 until May 2009. We collected and analyzed 4 patients with normal cervical tissue, 3 with cervical dysplasia, and 6 with cervical cancer. We only analyzed dysplasia samples in patients with pathologically confirmed CIN 2 or CIN 3 lesions. For patients with cervical cancer, we only analyzed patients with squamous cell carcinoma.

Patient characteristics, including pathology and treatment information, are shown in Table 3. Due to the small sample size, we included only those patients with cervical cancer or dysplasia that were found to be HPV 16 positive by PCR analysis (not shown). Based on the results of the qRT-PCR analysis, all HPV-16 samples were also found to express the E6 and E7 oncogenes (data not shown).

Based on the TaqMan® MicroRNA Array data from the cervical tissue samples, we found that 20 miRNAs were differentially expressed in cervical cancer compared to the normal cervical tissue at $p<0.05$. Of these, eighteen miRNAs were overexpressed and two were underexpressed. The fold change of various miRNAs ranged from 2.8 to 58.2. (Table 4) Unsupervised hierarchical clustering grouped the samples into two categories based on their miRNA expression, with the dysplasia samples grouping more

Table 3. Patient Characteristics

Sample	Specimen Number	Type	Patient Age	Diagnosis	Stage	Treatment
1	32	Benign	51	Uterine Prolapse	n/a	Hysterectomy
2	49	Benign	39	H/O Breast Cancer	n/a	Hysterectomy
3	167	Benign	35	Menorrhagia	n/a	Hysterectomy
4	367	Benign	50	Menorrhagia	n/a	Hysterectomy
5	79	Dysplasia	56	CIN 3	n/a	LEEP
6	80	Dysplasia	27	CIN 2	n/a	LEEP
7	83	Dysplasia	26	CIN 2	n/a	LEEP
8	33	Cancer	71	Squamous Cell Carcinoma	3B	XRT/Chemo
9	42	Cancer	40	Squamous Cell Carcinoma	2B	XRT/Chemo
10	47	Cancer	52	Squamous Cell Carcinoma	3B	XRT/Chemo
11	52	Cancer	61	Squamous Cell Carcinoma	1B1	Radical Hysterectomy
12	57	Cancer	38	Squamous Cell Carcinoma	1B2	Radical Hysterectomy
13	81	Cancer	34	Squamous Cell Carcinoma	1B1	Radical Hysterectomy

Table 4. MiRNAs differentially expressed in cervical cancer tissue compared to normal cervical tissue ($p<0.05$).

MiRNA	Fold change
Overexpressed	
hsa_miR_124	58.2
hsa_miR_449a	35.3
hsa_miR_449b	30.5
hsa-miR- 301b	24.3
hsa_miR_517c	22.4
hsa_miR_545	17.4
hsa_miR_223	14.2
hsa_miR_135b	11.1
hsa_miR_21	9.6
hsa_miR_512_3p	9.4
hsa_miR_542_3p	7.8
hsa_miR_181c	7.6
hsa_miR_517a	6.9
hsa_miR_518f	5.0
hsa_miR_106b	4.3
hsa_miR_192	3.8
hsa_miR_16	3.8
hsa_miR_141	2.8
Underexpressed	
hsa_miR_433	-18.6
hsa_miR_218	-5.6

closely with the normal cervical tissue than the cervical cancer tissue (Figure 5). The 18 miRNAs on the top are overexpressed in cervical cancer compared to the normal cervical tissue. Two miRNAs, shown at the bottom (miR-433 and miR-218), are underexpressed in cervical cancer compared to the normal cervical tissue. The 3 dysplasia samples (CIN79, CIN80, and CIN83) show differential expression of the twenty miRNAs (Figure 9).

To validate the miRNA data obtained from array analysis, we carried out individual qRT-PCR analysis of the 20 differentially expressed miRNAs. We identified 10 miRNAs that most significantly differed in the cancer tissues compared to the normal cervical tissues. The individual qRT-PCR expression plots for these differentially expressed miRNAs, showing relative quantification for each sample, are shown in Figure 10. We found that several miRNAs were generally overexpressed in cancer specimens compared to both normal and dysplasia tissue. This included miR-135b, miR-223, and miR-301b. A number of miRNAs such as miR-16, miR-21, miR-106b, miR-141, and miR-449a were generally overexpressed in both cancer and dysplasia specimens compared to the normal cervical tissue. MiR-218 and miR-433 were mostly underexpressed in the cancer and dysplasia tissues compared to the normal cervical samples. For many of the miRNAs such as miR-16, miR-21, miR-106b, miR-141, miR-449a (overexpressed) miR-218, and miR-433 (underexpressed), the dysplasia samples showed mixed results. Some of the dysplasia specimens such as CIN83, appeared more similar to the normal cervical tissue samples, while others such as CIN79 and CIN80, were more similar to the cancer tissue samples (Figure 10).

Figure 9. Unsupervised hierarchical clustering ($p<0.05$) of cellular miRNAs with increased (Ang et al.) or decreased expression (green) in cervical cancer tissue, cervical dysplasia, and normal cervical tissue, based on TaqMan® MicroRNA Array data.

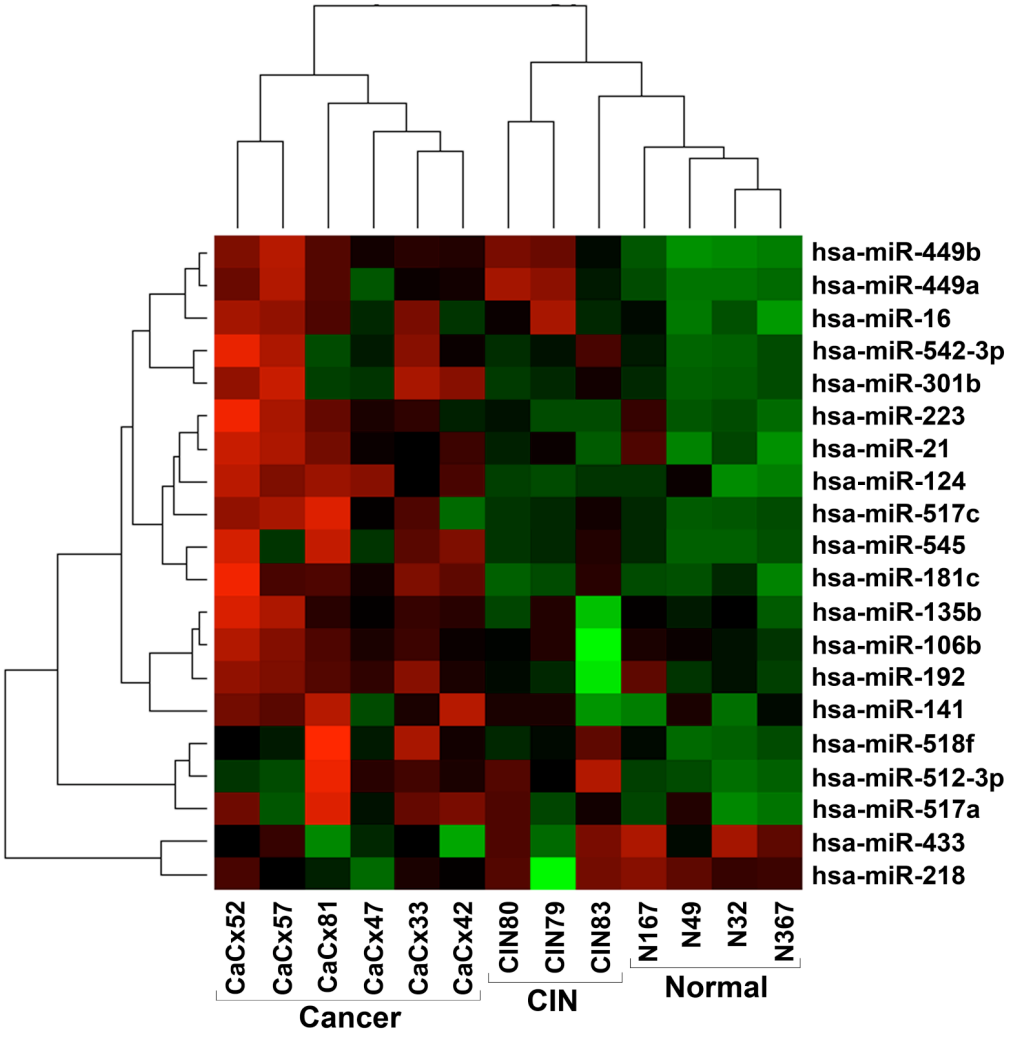
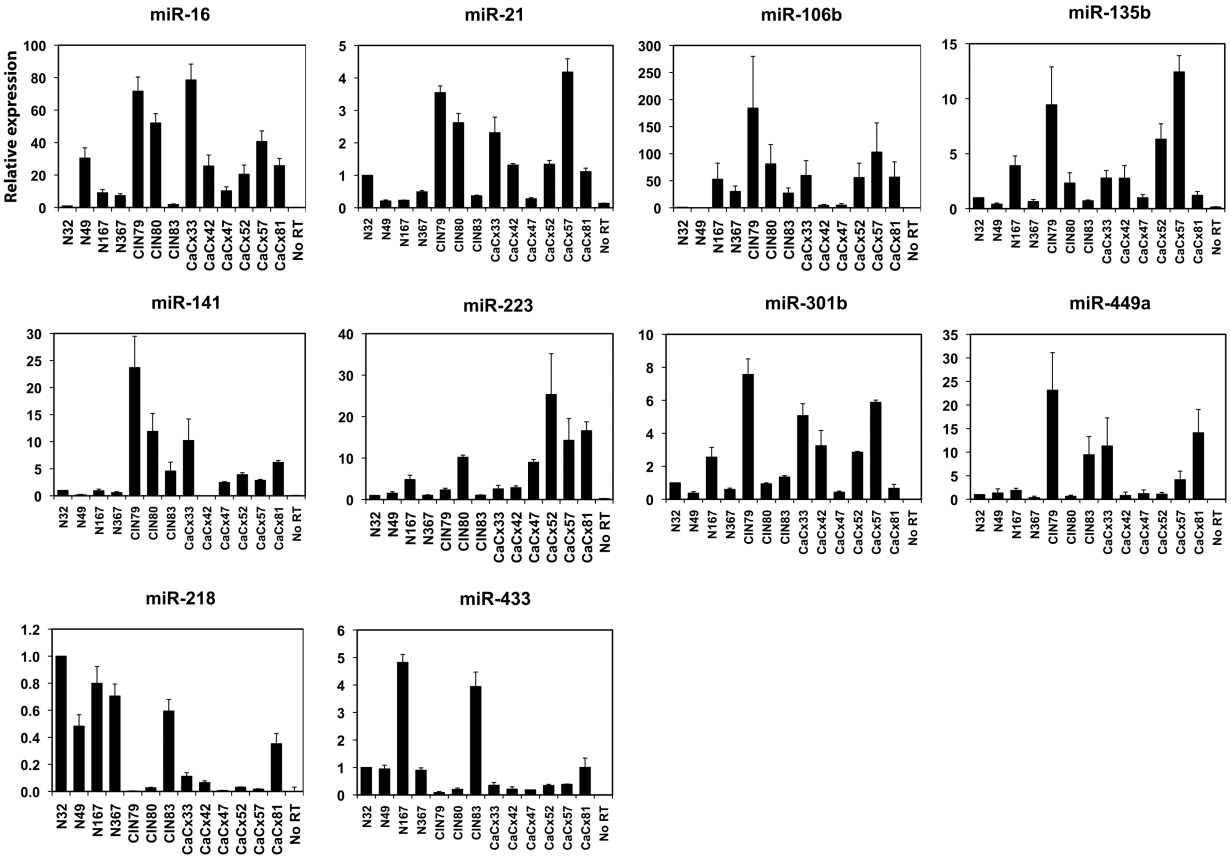


Figure 10. Individual qRT-PCR expression plots for miRNAs differentially expressed in normal cervical tissue, cervical dysplasia, and cervical cancer tissue.



We next sought to identify potential gene targets of the miRNAs we found differentially expressed in our tissue samples. We evaluated gene expression signatures reported by Gius *et al*. 2007, in which they utilized National Cancer Institute ROSP 8K human arrays to identify differentially expressed genes from the normal cervix, cervical dysplasias, and cervical squamous cell carcinomas from both epithelial and underlying stromal tissues (Gius et al., 2007). We compared this data with bioinformatically predicted gene targets from MicroCosm Targets for the miRNAs in our study. Table 5 displays potential gene targets for the ten miRNAs found to be most significantly over or under expressed in our analysis.

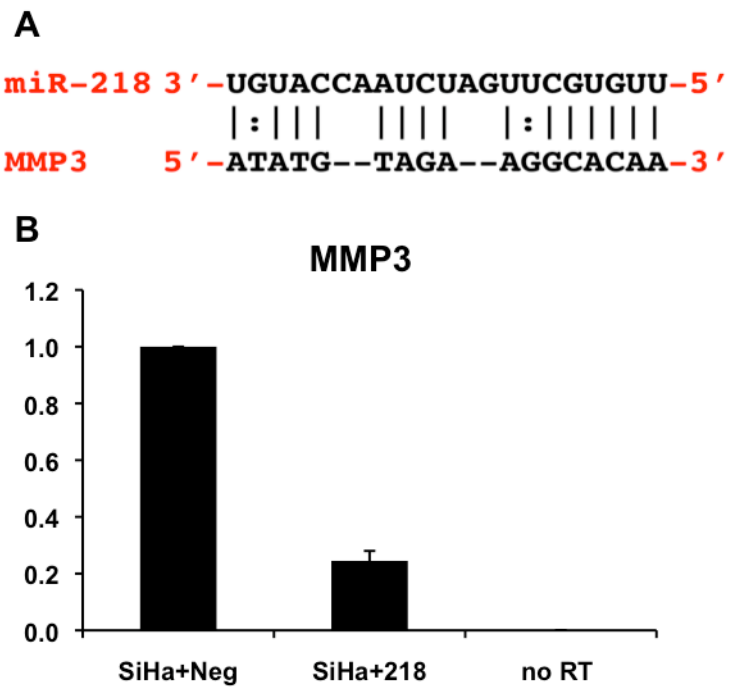
3.4 DISCUSSION

Changes in miRNA expression have been linked to many cancers. They are often found near fragile sites in chromosomes or near integration sites of high risk HPVs (Lee et al., 2002). MicroRNAs can either serve as tumor suppressor genes or oncogenes. Over expression of miRNAs that target oncogenes can lead to increased destruction of these oncogenes and therefore tumor suppression. Conversely, over expression of miRNAs that target tumor suppressors can result in increased oncogenic activity and tumor formation. MicroRNAs have been found to regulate the oncogenes *Bcl* and *Ras* as well as the tumor suppressor pRb (Volinia et al., 2006). MiR-15 and miR-16 were the first miRNAs shown to be associated with cancer; they were found to be down regulated in chronic lymphocytic leukemia (CLL) (Calin et al., 2002).

Table 5. Potential gene targets of miRNAs differentially expressed in cervical dysplasia and cancer. Computational targets of miRNAs differentially expressed in our study were obtained from MicroCosm and cross-referenced with genes that were also found to be differentially expressed in cervical dysplasia and cancer (Gius et al., 2007). Genes underexpressed in cervical dysplasia and cancer are potential targets of miRNAs that are overexpressed, and similarly, genes overexpressed in cervical dysplasia and cancer are potential targets of miRNAs that are underexpressed.

MiRNA	**Potential Targets**
Overexpressed	***Underexpressed***
miR-16	CDA, CDC37, NPC1, PPL, PVRL2, TGM1, ACAA1, PPAP2A, RBX1, UCP2, WNT7A
miR-21	ANXA1, CD48, DNAJA2, EIF4G2, EPHX1, IL13RA1, C2, DDAH1, PLA2G4A
miR-106b	DPAGT1, NINJ2, TBX19, ATP5F, FUT3, TPT1
miR-135b	ACAA1, DDX58, IL13RA1, SPINK5, AREG, HNRPA1, HNRPAB, NDUFA6
miR-141	EPHA2, NPC1, BRWD1, DR1, SELE
miR-223	DSG3, EIF4G2, BAG1, RBBP6
miR-301b	PPL, RASAL1, TBX1, TERF2, TGM1, C2, H19, RPS10, SELE
miR-449a	CDA, CDC37, NPC1, PPL, PVRL2, TGM1, AREG, ARHGBIB
Underexpressed	***Overexpressed***
miR-218	C1ORF41, RTCD1, SHMT2, SSR1, BAI3, CCNI, EBI2, HAS3, IGF2, MMP3, MTIG, PLAUR, PRIM2A, PSMB7, RGS1, RPS29
miR-433	GPR56, MRPL33, PABPC4, TARBP1, C1S, CD1A, CUL2, ISGF3G, NM1, RPS16, TRIP12

Figure 11. miR-218 reduces the expression of MMP3. (A) Binding site for miR-218 in the 3'UTR of MMP3. (B) qRT-PCR expression for MMP3 in SiHa cells transfected with a negative control precursor miRNA molecule or pre-miR-218. Standard deviation was calculated from three replicates ($p<0.05$).



MicroRNAs have been shown to negatively regulate the *Bcl-2* oncogene that is over expressed in many cancers (Cimmino et al., 2005). The *let-7* family of miRNAs regulates the *ras* oncogenes, which contain activating mutations in about 15-30% of cancers (Johnson et al., 2005). Downregulation of the *let-7* family of miRNAs results in the upregulation of *ras*, which is most pronounced in lung cancers (Takamizawa et al., 2004).

In the current study, we have demonstrated significant overexpression of 18 miRNAs and underexpression of two miRNAs. Similar to other work performed in the field, we specifically showed overexpression of miR-16 and miR-21, and underexpression of miR-218 in the cervical cancer tissues compared to normal cervical tissues (Martinez et al., 2008) (Lee et al., 2008). In addition to showing differential expression of miRNAs in cervical cancer tissue, this is one of the first published reports of differential expression of miRNAs in cervical dysplasia. We showed overexpression of miR-16, miR-21, miR-106b, miR-141, and miR-449a, and underexpression of miR-218 and miR-433. This differential expression was seen in the moderate/severe dysplasia samples as compared to the normal cervical samples. Other miRNAs showed similar expression patterns in dysplasia and normal cervix, such as miR-135b, miR-223, and miR-301b, which were generally overexpressed in cervical cancer samples.

Wang *et al.* identified several miRNAs via microarray that were deregulated in cervical cancer tissue (Wang et al., 2008). They found miR-15b, miR-16, miR-146a, and miR-155 to be highly overexpressed in cervical cancer tissues and miR-126, miR-143, and miR-145 to be underexpressed. In common with our study, they found that

miR-16, miR-21, miR-181c, and miR-223 were overexpressed and miR-218 was underexpressed (Wang et al., 2008). Using a cloning strategy to identify differentially expressed miRNAs, Lui *et al*. analyzed the respective cloning frequency of miRNAs in cervical carcinoma cell lines versus normal cervical tissue (Lui et al., 2007). They further evaluated the expression levels of miR-21 and miR-143 and found significant overexpression of miR-21 and underexpression miR-143 in cervical cancer cell lines and tissues versus normal cervical tissue (Lui et al., 2007). In previous research performed in our laboratory, Martinez *et al*. showed that miR-218 was significantly underexpressed in HPV-16 positive cervical cancer cell lines and tissues (Martinez et al., 2008).

Lee *et al.* analyzed 157 cellular miRNAs via the TaqMan® MicroRNA Human Early Panel Kit (Applied Biosystems) in 10 cervical cancer specimens and 10 normal cervical specimens. They found overexpression of 68 miRNAs and underexpression of two. They found that the 10 miRNAs that were most overexpressed were miR-199-s, miR-9, mir-199*, miR-199a, miR-199b, miR-145, miR-133a, miR-133b, miR-214, and miR-127. Only 2 miRNAs (miR-149 and miR-203) were found underexpressed. In common with our study, they found that miR-16, miR-21, miR-135b, and miR-181c were overexpressed. Of note, the cervical cancer tissues were not characterized by HPV type (Lee et al., 2008). Taken together, the miRNA expression patterns in cervical cancer that appear to be common in our study and those of others, are overexpression of miR-16 and miR-21 and underexpression of miR-218.

Many of the differentially expressed miRNAs from our study are differentially expressed in a variety of other cancers as well. MiR-16 is overexpressed in serous

ovarian cancer (Nam et al., 2008) as well as cancer of the head and neck (Hui et al.). Several researchers have shown overexpression of miR-21 in the following cancers: ovarian (Lui et al., 2007), head and neck (Hui et al.), lung (Zhang et al., 2010), stomach (Guo et al., 2008), liver (Jiang et al., 2008), and pancreas (Lee et al., 2007). Overexpression of miR-106b has been demonstrated in gastric (Guo et al., 2008) and colorectal cancer (Wang et al., 2010b), as well as squamous cell carcinoma of the head and neck (Hui et al.). MiR-135b was found overexpressed in colorectal (Wang et al., 2010b) (Bandres et al., 2006) (Sarver et al., 2009) and prostate (Tong et al., 2009) cancer. Nam *et al.* showed that overexpression of mir-141 was associated with a poor prognosis in patients with serous ovarian carcinoma (Nam et al., 2008). MiR-223 was shown to be overexpressed in adult T-cell leukemia (Bellon et al., 2009) and adenocarcinomas of the esophagus (Mathe et al., 2009). Conversely, miR-218 is underexpressed in a variety of cancers including breast, ovarian, and melanomas (Volinia et al., 2006) (Zhang et al., 2006). Lastly, Ueda *et al.* and Luo *et al.*, in separate studies, showed underexpression of miR-433 in gastric cancer (Ueda et al., 2010) (Luo et al., 2009).

The regulation of various gene targets by miRNAs has also been studied. Relevant to our study, Kaddar *et al.* demonstrated that miR-16 can negatively regulate genes that are involved in cell proliferation, such as HMGA1 and caprin-1 in MCF-7 and HeLa cells, but miR-16 induced G1 accumulation occurred only in MCF-7 cells, not in HeLa cells, which contain HPV-18 DNA integrated into the chromosome (Kaddar et al., 2009). The authors speculate that this is most likely due to the status of p53 and Rb, which function normally in MCF-7 cells but are inactivated in HeLa cells. Lack of miR-16

induced G0/G1 accumulation in HeLa cells was also observed by another group (Bandi et al., 2009). MiR-21 was found to downregulate the tumor suppressor PTEN in non-small cell lung cancer (Zhang et al., 2010), as well as in hepatocellular cancer (Meng et al., 2007). MiR-21 also targets the tumor suppressor gene tropomyosin 1 (TPM1) (Zhu et al., 2007), and the tumor suppressor genes programmed cell death 4 (PDCD4) and maspin, both of which have been implicated in tumor invasion and metastasis (Zhu et al., 2008). MiR-218 targets the OC-2 transcription factor during liver and pancreatic development (Simion et al., 2010). A recent study demonstrated that in gastric cancer miR-218, which is encoded in Slit genes, can target Robo1, providing a negative feedback loop through the Robo-Slit interaction (Tie et al., 2010).

We identified potential targets of miRNAs in cervical cancer (Table 5). Gene expression data from Gius *et al.* was cross-referenced with gene targets from MicroCosm Targets to identify a number of potential targets for the miRNAs in our study (Gius et al., 2007). WNT7A, a potential target for miR-16, is downregulated in cervical cancer (Gius et al., 2007) and has been shown to have an antitumorigenic effect in non-small cell lung cancer (Winn et al., 2006). MiR-21 may target annexin A1 (ANXA1) and EIF4G2, which are downregulated in cervical cancer (Gius et al., 2007). Reduced expression of ANXA1 is associated with advanced stage breast cancer (Wang et al., 2010a), and downregulation of EIF4G2 correlates with invasive transitional cell carcinoma of the bladder (Buim et al., 2005). Interestingly, matrix metalloproteinase 3 (MMP3), which has been shown by several groups to be upregulated in cervical cancer (Gius et al., 2007) (Hagemann et al., 2007), is a potential target of miR-218 (Figure 11).

Identification of a panel of miRNAs that can be used as early biomarkers in cervical cancer is potentially useful to determine disease behavior and prognosis. Targets of miRNAs may also provide new targets for anti-cancer therapy. The progression of invasive cervical cancer from mild, asymptomatic HPV infection to advanced stage invasive cervical cancer has been well documented. Although the majority of patients with moderate cervical dysplasia, even if untreated, will not progress to cervical cancer, a significant fraction will. MicroRNA expression profiling may enable us to identify those women with more aggressive disease and therefore provide more aggressive treatment for such patients.

4.0 CHAPTER 4

HUMAN PAPILLOMAVIRUS (HPV)-16 E6 REGULATES MICRORNA-218 VIA THE HISTONE ACETYLTRANSFERASE P300

4.1 INTRODUCTION

Human papillomaviruses are causative agents of cervical cancer (Walboomers et al., 1999). HPVs encode two oncogenes, E6 and E7, which serve to immortalize cells. E7 interacts with pRb family members (Chellappan et al., 1992), resulting in the expression of S phase genes but also activation of the p53 stress response (Moody and Laimins, 2010). E6 then targets p53 in complex with E6AP (Werness et al., 1990) (Huibregtse et al., 1991), resulting in p53 ubiquitination and degradation (Scheffner et al., 1990). E6 interacts with a number of other cellular proteins, including histone acetyltransferases p300/CBP (Patel et al., 1999) (Zimmermann et al., 1999). This interaction prevents acetylation of p53, further preventing p53-mediated transcription.

MicroRNAs are ~22 nt single-stranded gene regulators, which most often function to repress translation or degrade mRNA transcripts (Kim, 2005). They are important in cellular development and differentiation, and deregulation of miRNAs has been reported for many cancers (Calin and Croce, 2006a). In cervical cells, miR-34a and miR-203 are targets of p53. MiR-34a regulates CDK4 and CCNE2, and directly targets E2F3 and SIRT1 (Vecchione and Croce, 2010). MiR-203 regulates p63 (McKenna et al., 2010). We previously showed that miR-218 is underexpressed in HPV-16 positive cervical carcinoma cells, and HPV-16 E6 specifically downregulates miR-218 (Martinez et al., 2008). MiR-218 in turn targets LAMB3, a component of the basement membrane protein laminin5, which can serve as a transient receptor for HPV (Martinez et al., 2008). We also showed that miR-218 is underexpressed in HPV-16

positive cervical dysplasias and cancer (see Chapter 3). In this study, we sought to understand the mechanism of regulation of miR-218 by HPV-16 E6. We found that E6 regulates miR-218 through the histone acetyltransferase p300, and this function is not dependent on p53. We also found that miR-218 reduces the migration and invasion of cervical carcinoma cells.

4.2 MATERIALS AND METHODS

Cell culture, transfections, and infections

LXSN vectors encoding wild-type HPV-16 E6 and the E6 mutants L50G and G130V were obtained from Dr. Elliot Androphy (Indiana University). HEK293 cells were seeded $2x10^6$ in 10 mm plates and transfected the next day with 5 μg ampho plasmid and 10 μg LXSN DNA. After 48-72 hrs, the viral supernatant was collected, filtered, and added to U2OS cells. Following an additional 48 hr, the cells were harvested and RNA extracted using the Trizol reagent (Invitrogen). Cervical cancer cell line SiHa (HPV-16 positive) was seeded 1 $x10^5$ in 12-well plates and transfected the next day with 250 nM siRNA molecules targeting EZH2 (Dharmacon) using Lipofectamine 2000 (Invitrogen). The HPV-negative cervical cancer cell line C-33A was also seeded 1 $x10^5$ and transfected the next day with 250 nM siRNA molecules targeting p300 (Dharmacon) using Lipofectamine 2000 (Invitrogen). Fluorescent BLOCK-iT was used to check transfection efficiency (Invitrogen). After 48 hr, the cells were harvested and RNA extracted with Trizol (Invitrogen).

Drug treatments

SiHa cells were seeded 1 x10^5 in 12-well plates and treated the next day with either DMSO, 5 μM 5-azacytidine, 0.5 μM trichostatin A, 50 μM adenosine dialdehyde, or a combination. C-33A cells were seeded 1 x10^5 in 12-well plates and treated the next day with either DMSO or 30 μM anacardic acid.

MicroRNA and mRNA Real-Time Quantitative RT-PCR Analysis

Total RNA (10 ng) was subjected to qRT-PCR analysis using the TaqMan® miRNA Reverse Transcription Kit and miRNA Assays (Applied Biosystems, USA), and the Real-Time thermocycler iQ5 (BioRad, USA). The small nucleolar RNU43 was used as the housekeeping small RNA reference gene for miR-218. For qRT-PCR analysis of SLIT2, SLIT3, p300, and EZH2 mRNAs, 300 ng of total RNA was amplified using the one-step QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, USA). The following primers were used: (SLIT2, forward 5'-CTGTGAATGCAGCAGTGGAT-3' and reverse 5'-TTGTTTGGCAAGCAGCATAG-3' (116-bp product); SLIT3 forward 5'-GCGCCTGAACAAGAATAAGC-3' and reverse 5'-GATCCCCTGGATCTGGTTTT-3' (103-bp product; p300 forward 5'-TAAACTCTCATCTCCGGCCC-3' and reverse 5'-CCACCATTGGTTAGTCCCAA-3' (129-bp product; EZH2 forward 5'-GGAACAACGCGAGTCGG-3' and reverse 5'-CTGATTTTACACGCTTCCGC-3' (102-bp product). The primer sequences for the control housekeeping glyceraldehyde-3-

phosphate dehydrogenase gene (G3PDH) have been described previously (Martinez et al., 2007). All reactions were done in triplicate and relative expression of RNAs was calculated using the 2 delta CT method (Livak and Schmittgen, 2001).

In vitro migration and invasion assays

SiHa cells were seeded 2 x10^5 in 6-well plates and transfected the next day with either 100 nM negative control precursor miRNA molecules or miR-218 precursor molecules (Ambion) using Lipofectamine 2000 (Invitrogen). FAM-labeled precursor molecules were used to check transfection efficiency (Ambion). After 72 hr, migration assays were performed, and the cells were seeded onto 24-well Transwell inserts, 8 μm pore size (Corning), in media without serum. The lower chambers of the Transwell plate were filled with DMEM containing 20% FBS to serve as a chemoattractant. For invasion assays, the Transwell inserts were coated with Matrigel basement membrane extract (BD Biosciences). After 24 hr, migratory or invasive cells in the membrane were fixed with 100% methanol and then stained with 0.1% crystal violet. The cells were then counted under a microscope at 400X magnification. The mean of five fields from six separate assays was used.

In vitro wound-healing assays

SiHa cells were seeded 2 x10^5 in 6-well plates and transfected the next day with either 100 nM negative control precursor miRNA molecules or miR-218 precursor

molecules (Ambion) using Lipofectamine 2000 (Invitrogen). FAM-labeled precursor molecules were used to check transfection efficiency (Ambion). After 48 hr, the cells were seeded 2 x10^5 into 24-well plates and allowed to become confluent overnight. The wells were then scratched with a sterile pipette tip creating a lesion approximately 500 μm in width. The wells were photographed and the wound gap was measured. After 24 hr, the gap was photographed and measured again. Six independent experiments were performed.

4.3 RESULTS

HPV-16 E6 regulates miR-218 through p300

Previously we found that HPV-16 E6 reduces the levels of miR-218 in cervical carcinoma cells (Martinez et al., 2008). In order to identify the mechanism by which E6 regulates miR-218, we utilized LXSN vectors encoding either wild-type E6 or mutant E6. We obtained the well-characterized mutants L50G, which does not bind E6AP or p53, and does not degrade p53, as well as G130V, which shares the same properties as L50G but has reduced ability to bind p300/CBP (Sekaric et al., 2008) (Table 6). The G130V mutation is located in the second zinc-finger domain of E6 that is essential for p300/CBP binding (Patel et al., 1999). The HPV-16 E6 and p300 studies were carried out in U2OS cells (Patel et al., 1999) (Zimmermann et al., 1999). We found that in U2OS cells wild-type E6 reduced miR-218 as previously shown, as did the mutant

Table 6. HPV-16 E6 mutant properties. (Patel et al., 1999)

Mutant	Bind E6AP	Bind E6BP	Bind p53	Degrade p53 at 25°	Degrade p53 at 37°	p53 levels at early passage	p53 levels at late passage	Immortalize MECs	Activate hTERT	Degrade hADA3	Bind CBP/p300
WT E6	+++	+++	+++	+++	+++	low	low	yes	yes	yes	yes
L50G	-	-	-	-	-	normal	none	no	no	no	yes
G130V	-	-	-	-	-	normal	none	no	no	no	no
+++ 51-100% activity, ++ = 21-50% activity, + = 6-20% activity, +/- = 1-5% activity, - = <1% activity											

L50G, when compared to the empty vector (Figure 12B). However, no reduction in miR-218 levels was observed in the presence of the G130V mutant (Figure 12B). These results suggest that E6 regulates miR-218 through p300. We next transfected the HPV-negative cervical carcinoma C-33A cell line, which expresses high levels of miR-218, with siRNA molecules against p300 (Figure 12C). MiR-218 expression was reduced upon knock-down of p300 (Figure 12F). In addition, SLIT2 and SLIT3 were also reduced (Figure 12 D and E).

Histone 3 lysine 27 acetylation activity is essential for miR-218 expression

P300 is a histone acetyltransferase and competes with the histone methyltransferase EZH2 for modification of H3K27 in embryonic stem cells (Pasini et al., 2010). EZH2 was recently shown to bind to the SLIT2 promoter and inhibit its expression in prostate cancer cells (Yu et al., 2010). We sought to determine if EZH2 might similarly regulate the SLIT genes in cervical cancer cells. We transfected SiHa cells, which do not express miR-218, with siRNA molecules against EZH2 (Figure 13A). Although SLIT2 expression did not change significantly, SLIT3 and miR-218 expression increased upon knock-down of EZH2 (Figure 13B, C, and D).

We next treated C-33A cells with anacardic acid (AA), which inhibits the acetyltransferase activity of p300 and reduces H3K27 acetylation (Pasini et al., 2010). SLIT2 expression was not reduced, but both SLIT3 and miR-218 expression was reduced after 72 hr of AA treatment (Figure 14 A, B, and C). We also treated SiHa cells with adenosine dialdehyde (AdOx), which inhibits EZH2 and reduces H3K27

methylation (Miranda et al., 2009). SLIT2 expression did not increase, but both SLIT3 and miR-218 expression increased after 72 hr of AdOx treatment (Figure 14 D, E, and F).

The SLIT2 and SLIT3 promoters are methylated in cervical cancer (Narayan et al., 2006). However, Narayan *et al*. were unable to reactivate expression of either SLIT gene in SiHa and other HPV-positive cervical cancer cell lines following treatment with the DNA methyltransferase inhibitor 5-azacytidine (5-aza) or the histone deacetylase inhibitor trichostatin A (TSA) either alone or in combination. We treated SiHa cells with 5-aza, TSA, AdOx, or a combination and observed reactivation of SLIT2, SLIT3, and miR-218 expression after 72 hr (Figure 15). These results indicate that H3K27 acetylation at the SLIT2/3 promoters is important for SLIT and miR-218 expression.

MiR-218 reduces the migration and invasion of HPV-16 positive cervical carcinoma cells

In order to understand the function of miR-218, we performed a number of studies to identify how it might affect the cellular phenotype. We performed cell viability/cytotoxicity, proliferation, apoptosis, and cell cycle assays. We did not observe any significant differences between cells transfected with negative precursor molecules or miR-218 for these assays (data not shown). SLIT2 and SLIT3 are extracellular signaling molecules and have been shown to decrease the migration of cancer cells (Tseng et al., 2010). We therefore surmised that miR-218 might induce a similar phenotype. We performed Transwell migration and Matrigel invasion assays and found that miR-218 significantly reduced the migration and invasion of SiHa cervical

carcinoma cells (Figure 16). We also performed *in vitro* wound-healing assays and found that miR-218 reduces the ability of SiHa cells to close the gap lesion (Figure 17). Finally, we found that miR-218 transfected cells were more adhesive and had reduced capacity to grow in non-anchorage dependent conditions (data not shown).

Figure 12. p300 regulates miR-218. (A) HPV-16 E6 expression in U2OS cells infected with LXSN 16E6 WT or mutant constructs. (B) miR-218 expression in U2OS cells infected with 16E6 WT or mutant constructs. (C) p300 expression in C-33A cells transfected with siRNA molecules targeting p300. (D) SLIT2 expression in C-33A cells. (E) SLIT3 expression in C-33A cells. (F) miR-218 expression in C-33A cells.

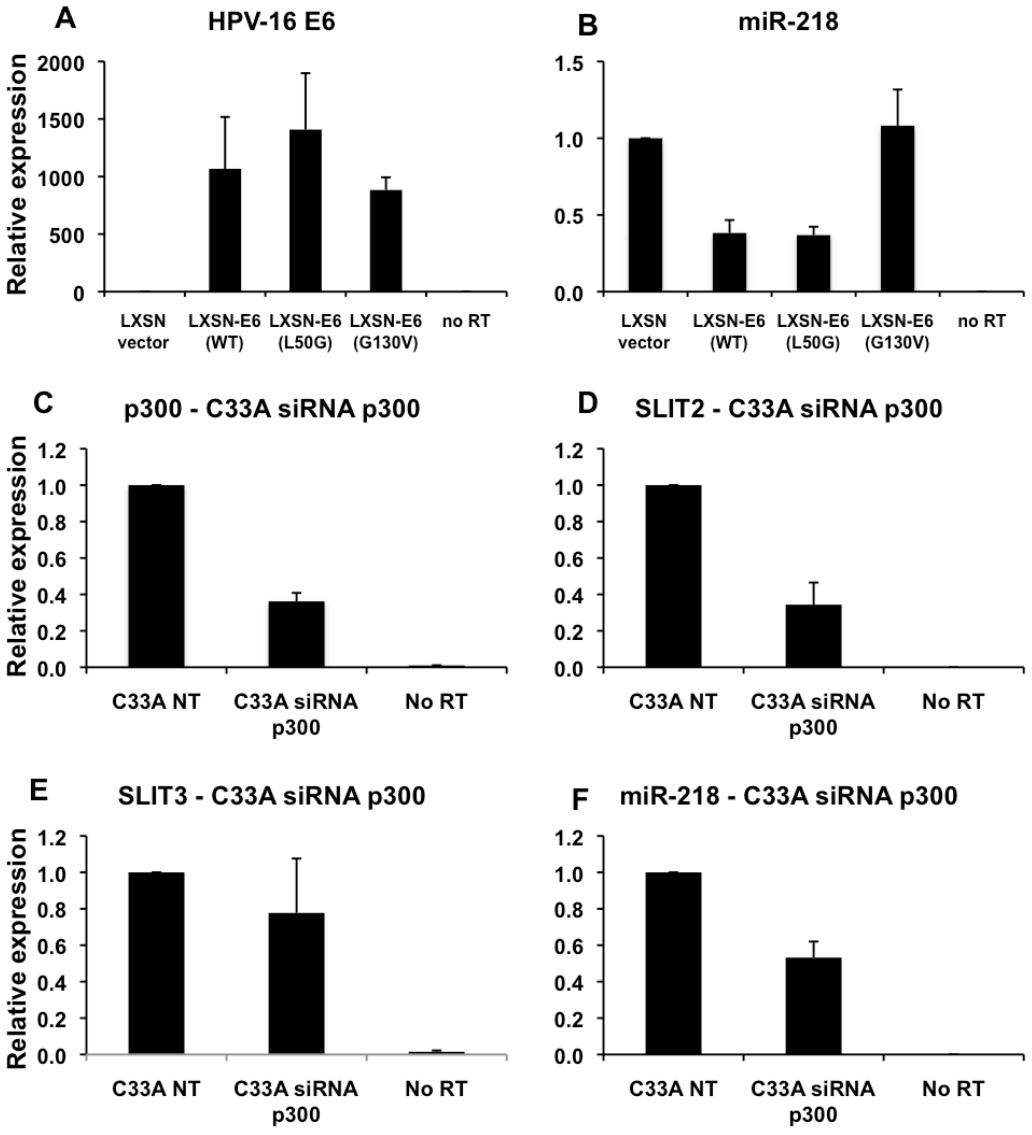


Figure 13. Reduction in EZH2 increases SLIT2, SLIT3, and miR-218 levels. (A) EZH2 expression in SiHa cells transfected with siRNA molecules targeting EZH2. (B) SLIT2 expression. (C) SLIT3 expression. (D) miR-218 expression.

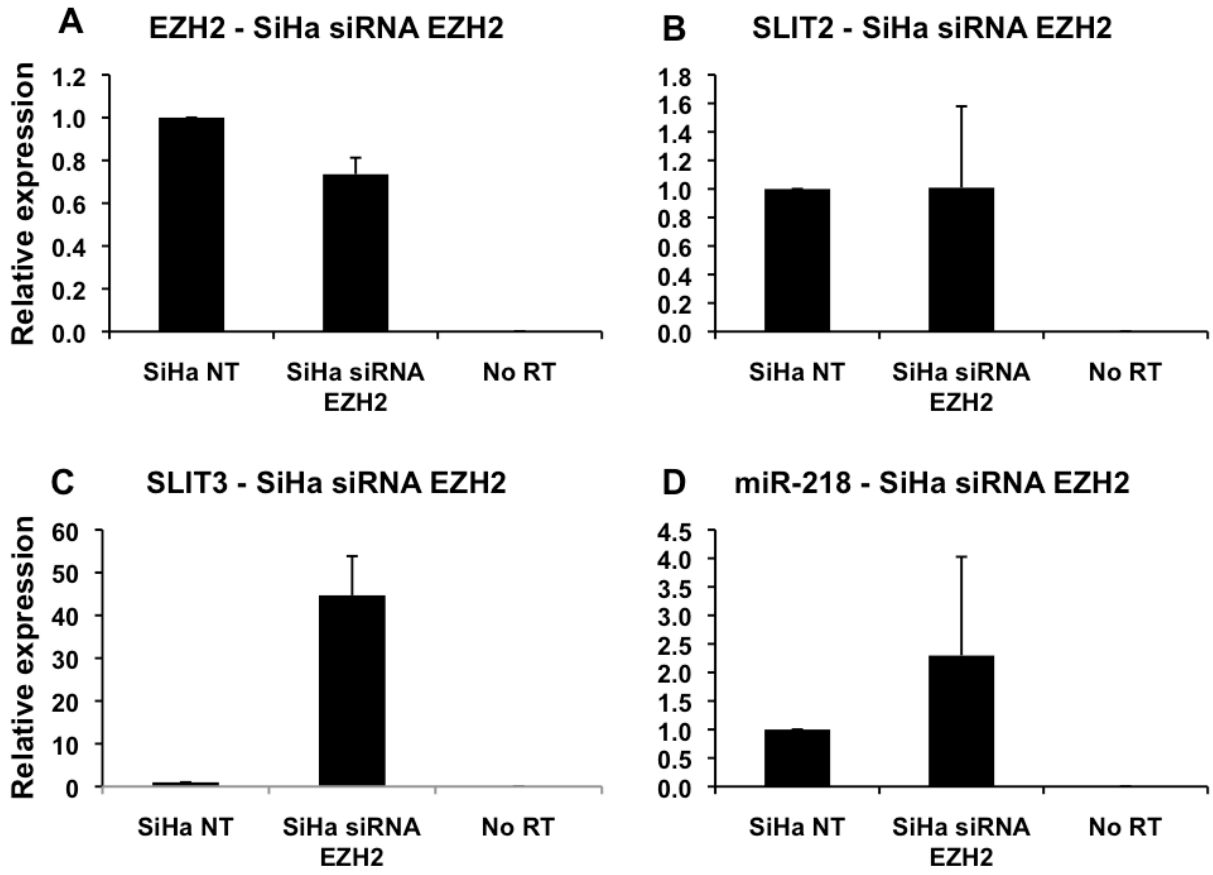


Figure 14. SLIT2, SLIT3, and miR-218 expression in cells treated with anacardic acid or adenosine dialdehyde. C-33A cells were treated with anacardic acid (AA) for 72 hr. (A) SLIT2 expression. (B) SLIT3 expression. (C) miR-218 expression. SiHa cells were treated with adenosine dialdehyde (AdOx) for 72hrs. (D) SLIT2 expression. (E) SLIT3 expression. (F) miR-218 expression.

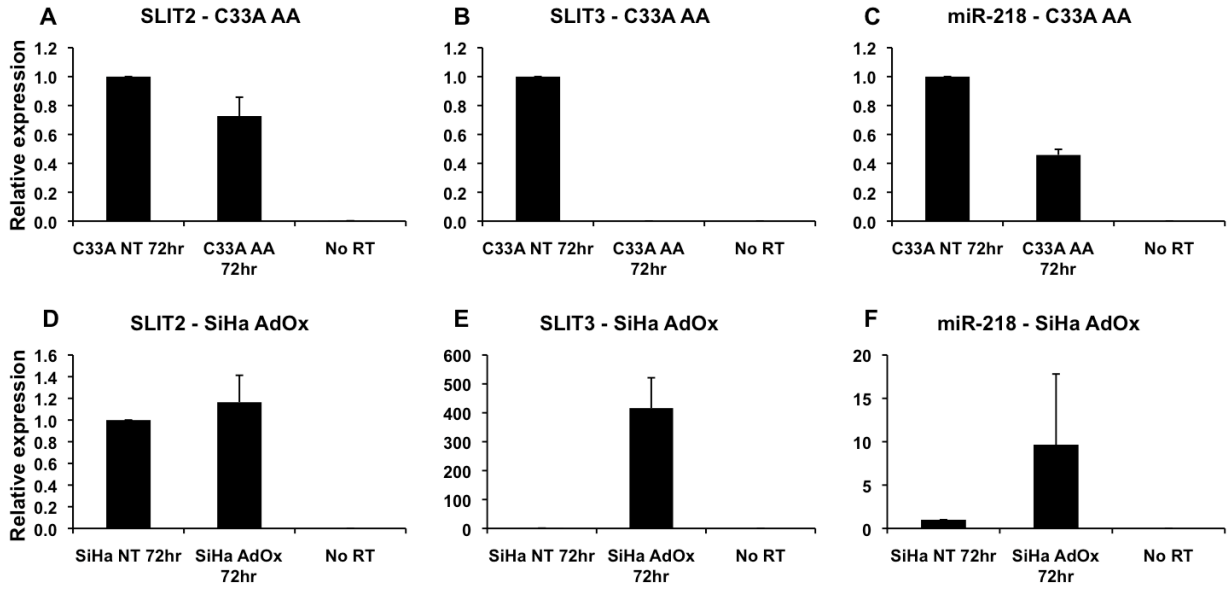


Figure 15. SLIT2, SLIT3, and miR-218 are increased upon treatment with a combination of 5-azacytidine, trichostatin A, and adenosine dialdehyde. SiHa cells were treated with 5-azacytidine (5-aza), trichostatin A (TSA), adenosine dialdehyde (AdOx), or a combination for 72 hr. (A) SLIT2 expression. (B) SLIT3 expression. (C) miR-218 expression.

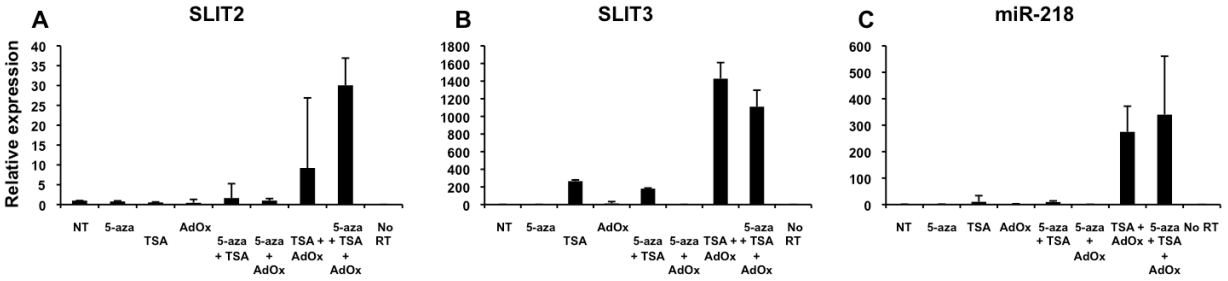


Figure 16. MiR-218 reduces the migration and invasion of SiHa cells. SiHa cells were transfected with a negative precursor miRNA molecule or pre-miR-218 for 72 hr. They were then seeded on transwell membranes that were either uncoated or coated with Matrigel basement membrane extract. After 24 hr, non-migratory cells were removed and the migratory cells in the membrane were stained with crystal violet. Six independent experiments were performed, and five fields/membrane were counted. $p<0.05$.

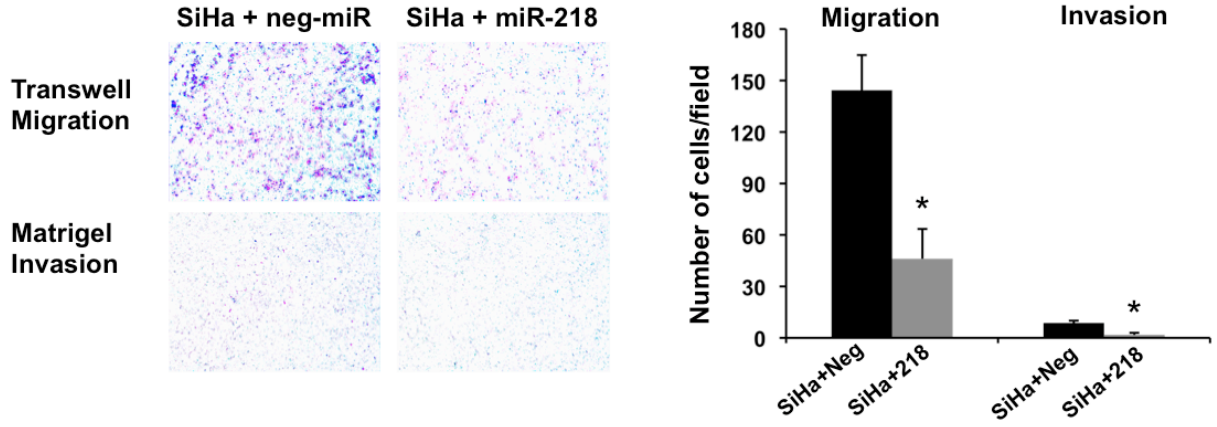
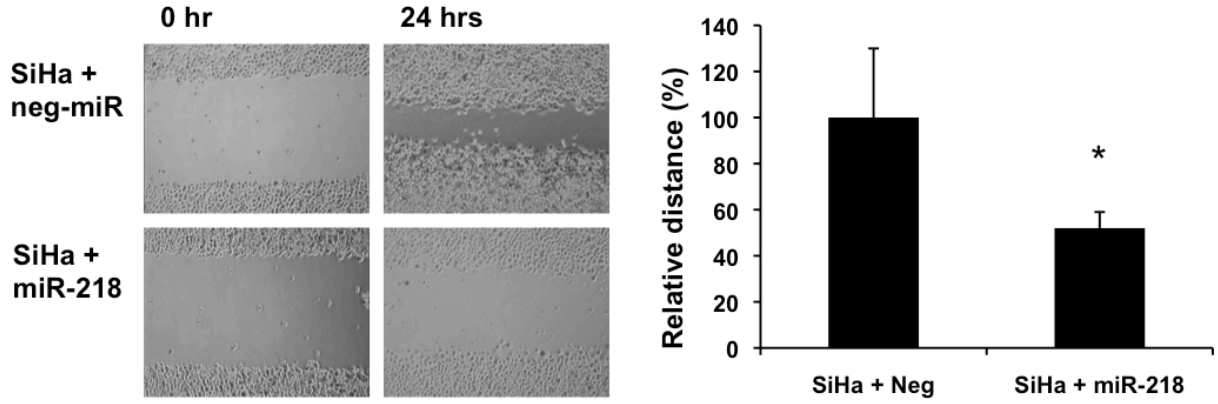


Figure 17. MiR-218 reduces the in vitro wound-healing capacity of SiHa cells. SiHa cells were transfected with a negative precursor miRNA molecule or pre-miR-218 for 72 hr. The wells were then scratched with a sterile pipette tip and photographed at 0 hr and 24 hr post-scratch. Six independent experiments were performed. The gap width/distance migrated was measured for each experiment. $p<0.05$.



4.4 DISCUSSION

We utilized the wild-type HPV-16 E6 protein and its E6 mutants to determine the mechanism by which E6 downregulates miR-218. We found that the G130V mutant, which is defective in p300-binding, did not reduce miR-218 expression (Figure 12). We further found that knock-down of p300 and inhibition of its acetyltransferase activity decreases SLIT2, SLIT3, and miR-218 (Figures 12 and 14).

In addition, knock-down and inhibition of EZH2 increases miR-218 levels (Figures 13 and 14). EZH2 is a polycomb group protein and functions in the polycomb repressive complex 2 (PRC2). PRC2 proteins EZH2 and SUZ12, and PRC1 proteins BMI1, RING1, and RING2, can bind to the SLIT2 promoter (Yu et al., 2010). PRC1 and PRC2 are transcriptional repressors that antagonize developmental regulators in embryonic stem cells. EZH2 catalyzes the dimethylation and trimethylation of H3K27, while p300 catalyzes the acetylation of H3K27 (Pasini et al., 2010). EZH2 has been reported to be overexpressed in various cancers (Varambally et al., 2002) (Wagener et al., 2008). Interestingly, HPV-16/18 E7 can increase EZH2 expression in cervical cancer cells (Holland et al., 2008), thus providing an additional mechanism by which high-risk HPVs may target miR-218. In our study, p300 acetyltransferase activity was necessary for miR-218 expression. We propose that p300 positively regulates miR-218 expression in normal cervical cells. Upon HPV-16 infection, E6 binds p300 and prevents acetylation of H3K27, allowing EZH2 to occupy the SLIT2/3 promoters, methylate H3K27, and prevent miR-218 expression (Figure 18).

SLIT2 and SLIT3 are large genes. SLIT2 is ~365 kb, and SLIT3 is ~635 kb in size. SLIT2 harbors miR-218-1 in intron 14, and SLIT3 harbors miR-218-2 in intron 15 (Figure 18). These miR-218 copies are identical. Because the distance between the transcriptional start sites of SLIT2/3 and miR-218-1/2 is large, it is possible that miR-218-1/2 have their own promoters. However, our work and that of others suggest that miR-218-1/2 are processed from the SLIT2/3 transcripts. We previously showed that SLIT2 and miR-218 are coordinately expressed in cervical cancer (Martinez et al., 2008). Others have shown that they are regulated similarly as well (Tie et al., 2010). In this study, we found that miR-218 expression closely matches the expression of SLIT2 and SLIT3. Although SLIT2 did not respond as well as SLIT3 to knock-down of EZH2 and AdOx treatment, all three behaved similarly when treated in combination with 5-aza, TSA, and AdOx (Figure 15). The SLIT2 promoter is hypermethylated in cervical carcinoma cells (Narayan et al., 2006), which may account for the greater requirement of a combination of the DNA and histone modifying drugs.

MiR-218 is underexpressed in many cancers including cervical, ovarian, and melanomas (Volinia et al., 2006) (Zhang et al., 2006). We showed that miR-218 reduces the migration and invasion of cervical carcinoma cells (Figure 16). MiR-218 also reduced the *in vitro* wound-healing capacity of cervical carcinoma cells (Figure 17). Others have reported similar findings (Tie et al., 2010) (Song et al., 2010). Based on the above results, our work supports the mounting evidence that miR-218 is a tumor suppressor miRNA in many cancers.

Figure 18. Location of miR-218-1 and miR-218-2 within SLIT2 and SLIT3. MiR218-1 is located within intron 14 of SLIT2. MiR-218-2 is located within intron 15 of SLIT3. (Tie et al., 2010)

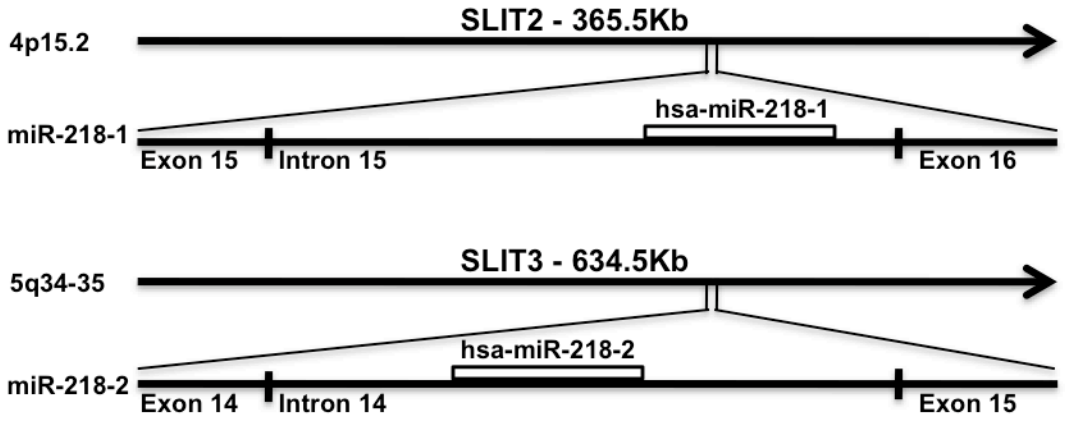
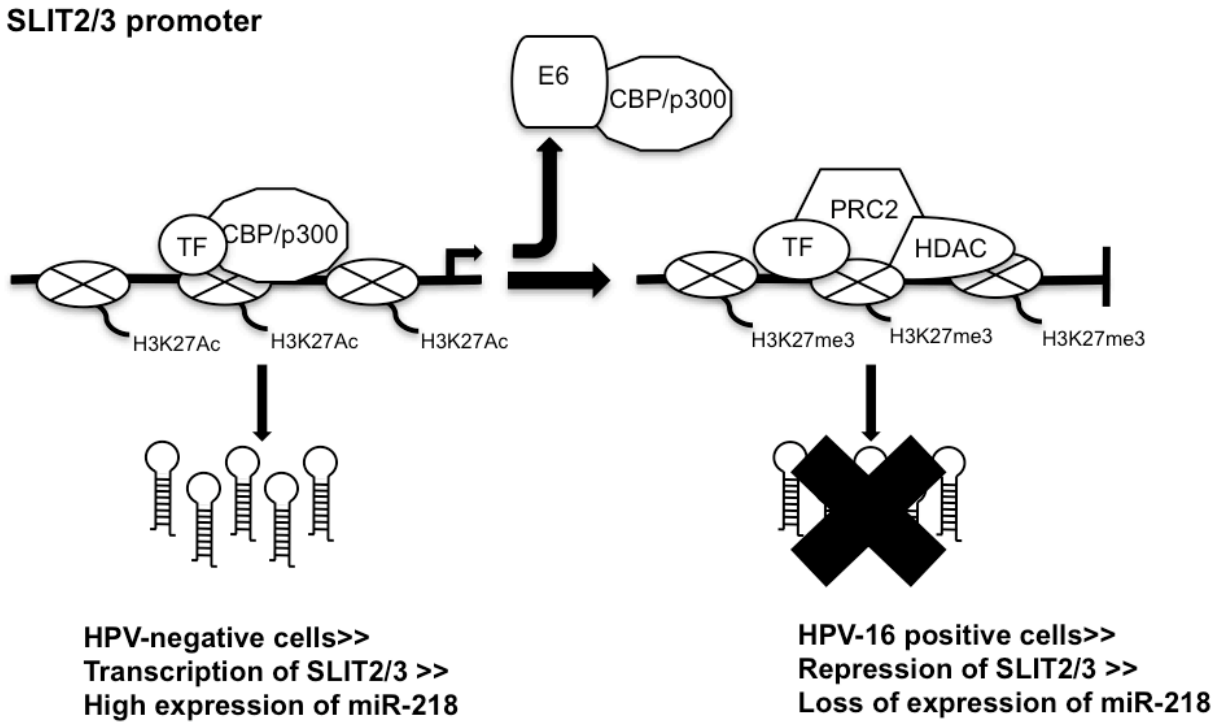


Figure 19. Model of miR-218 regulation. In cervical cells, p300 positively regulates SLIT2 and SLIT3, as well as miR-218-1/2, which are encoded by the introns of these genes. Upon HPV-16 infection, E6 binds to and reduces the HAT activity of p300, allowing the repressive histone methyltransferase EZH2, a component of the polycomb repressive complex (PRC2), to bind to the SLIT promoters and prevent miR-218 expression.



5.0 CHAPTER 5

GENERAL DISCUSSION

5.1 GENERAL DISCUSSION

In these studies, we have examined cellular microRNA expression in HPV-16 positive cervical carcinoma cells. We reported the basal expression level of cellular miRNAs in the normal cervix, and we analyzed the expression of cellular miRNAs in HPV-16 positive cervical cancer cell lines and tissues compared to the normal cervix. We demonstrated that HPV-16 E6 downregulates miR-218 in cervical cancer cells, and we identified LAMB3, part of the epithelial-specific protein laminin 5, as a miR-218 target. Downregulation of miR-218 allows overexpression and deposition of laminin 5 in the extracellular matrix where it can serve as a transient receptor for HPVs.

We further examined cellular miRNAs in cervical dysplasias and cancer, and found that miR-218 was underexpressed in these HPV-16 positive cervical dysplasia and cancer tissues as well. We provided expression profiles that may be useful as biomarkers. We identified MMP3 as an additional target of miR-218. MMP3 is an extracellular matrix protein that is overexpressed in cervical cancer (Hagemann et al., 2007) (Gius et al., 2007), and it is important for the epithelial-mesenchymal transition, regulating tumor cell motility and metastasis (Kessenbrock et al., 2010).

Finally, we demonstrated that HPV-16 E6 regulates miR-218 via the histone acetyltransferase p300 in cervical carcinoma cells. We found that p300 knock-down or p300 inhibition with anacardic acid reduced miR-218 expression in HPV-negative cells, and EZH2 knock-down or its inhibition with adenosine dialdehyde reactivated miR-218 expression in HPV-16 positive cells. Reactivation was enhanced upon treatment with a

combination of 5-azacytidine, trichostatin A, and adenosine dialdehyde. We also demonstrated that miR-218 reduces the migration and invasion of cervical cancer cells, indicating that miR-218 is a tumor suppressor with potential therapeutic value.

5.2 FUTURE DIRECTIONS

Future miR-218 directions will include studies to demonstrate binding of p300 and EZH2 to the SLIT2/3 promoters in cervical carcinoma cells. Although we showed that p300 regulates miR-218 expression and antagonizes EZH2, it may not bind directly to the SLIT2/3 promoters. ChIP assays could be performed to answer this question. Additional studies to identify transcription factors and other key players in this pathway may also be performed.

Analyses to identify additional cellular targets of miR-218 will be performed. SiHa cells were transfected with miR-218, and Argonaute 2-containing microribonucleoprotein particles (miRNPs) were immunoprecipitated in order to enrich for miR-218 and its targets. We eluted the RNA and are in the process of analyzing the sample compared to control samples from cells that were not transfected or transfected with a negative control molecule via microarray analysis.

Experiments to identify potential HPV-16 encoded miRNAs are also in progress. Viral miRNAs may serve as an efficient method for regulating both viral and cellular gene expression. Viral miRNAs were first identified in herpesviruses such as EBV, HCMV, KSHV, and HSV-1, which are large DNA viruses characterized by their latent life

cycles (Pfeffer et al., 2005) (Cai et al., 2005) (Cui et al., 2006). Polyomaviruses such as SV40, JC, and BK viruses are small double-stranded DNA viruses similar to HPVs, and they also encode miRNAs (Sullivan et al., 2005). Many of the miRNA-encoding viruses are associated with cancer, but to date no HPV-derived miRNAs have been identified.

We cultured normal immortalized human keratinocytes (NIKS) in an organotypic raft system that mimics epithelial tissue. Cells were seeded over a layer of collagen and fibroblast feeders assembled on a mesh raft in enriched media. We maintained twelve rafts each of HPV-16 infected and non-infected keratinocytes in this manner for ten days. This allowed the highly differentiation-dependent HPV-16 to replicate as the cells formed differentiated epithelial tissue. The tissues were harvested after ten days allowing sufficient time for cellular differentiation and viral gene expression to occur. An aliquot of total cell lysates was used to confirm expression of the HPV-16 E6 and E7 oncogenes in the HPV-positive rafts. We then immunoprecipitated Argonaute 2-associated miRNP particles using an antibody specific for human Ago2. This step greatly reduced the background of non-miRNA small RNAs and RNA breakdown products. We eluted RNA from these miRNP particles and confirmed the presence of miRNAs by quantitative RT-PCR of let-7 and miR-21. We expect that the small RNAs described in HPV-16 infected NIKS will be enriched in HPV-16 encoded miRNAs and will also contain any target mRNAs hybridized due to complementarity. The RNA fraction may also contain cellular miRNAs and their target mRNAs. We have isolated the above RNA fractions from both HPV-16 infected and uninfected cells. The GPCL at the University of Pittsburgh has performed deep sequencing of these samples using the SOLiD™ 4 System and data analysis is currently in progress. The discovery of HPV-

encoded miRNAs will increase our knowledge of the oncogenic properties of HPV-16, and such miRNAs may also have potential use in the early diagnosis, prognosis and treatment of cancer.

APPENDIX A

SUPPLEMENTARY TABLES

Supplementary Table 1. Basal expression of miRNAs in the normal cervix

Human miRNA	Median
hsa_miR_145	12003.5
hsa_miR_26a	9015.0
hsa_miR_99a	5937.5
hsa_let_7a	5205.5
hsa_miR_143	5135.0
hsa_let_7b	5034.5
hsa_let_7c	4859.0
hsa_miR_125b	4093.0
hsa_miR_126	3713.5
hsa_miR_195	3662.5
hsa_miR_21	3223.5
hsa_miR_24	3187.5
hsa_miR_23b	3131.5
hsa_let_7d	3032.5
hsa_miR_23a	2995.5
hsa_miR_16	2777.5
hsa_miR_100	2596.5
hsa_miR_27b	2387.0
hsa_let_7g	2332.0
hsa_miR_29a	2195.0
hsa_let_7f	2051.5
hsa_miR_107	1490.0
hsa_miR_125a	1401.5
hsa_miR_103	1392.5
hsa_miR_221	1374.0
hsa_let_7i	1369.0
hsa_miR_199a	1309.0
hsa_miR_191	1298.0
hsa_miR_199a_AS	1275.5
hsa_miR_205	1119.5
hsa_miR_30d	1074.5
hsa_miR_22	1049.0
hsa_miR_30b	1001.5
hsa_miR_30a_5p	996.5
hsa_let_7e	905.0
hsa_miR_10b	812.5
hsa_miR_222	775.5
hsa_miR_203	747.0
hsa_miR_26b	735.0
hsa_miR_130a	698.0
hsa_miR_30c	693.0
hsa_miR_152	652.0
hsa_miR_497	612.0

hsa_miR_148a	605.0
hsa_miR_368	597.5
hsa_miR_99b	554.0
hsa_miR_214	541.0
hsa_miR_200c	506.5
hsa_miR_106a	492.5
hsa_miR_27a	476.5
hsa_miR_30e_5p	458.5
hsa_miR_342	449.5
hsa_miR_10a	427.0
hsa_miR_19b	408.5
hsa_miR_17_5p	402.0
hsa_miR_31	399.0
hsa_miR_34a	394.0
hsa_miR_320	386.0
hsa_miR_133a	378.5
hsa_miR_200b	350.5
hsa_miR_15b	348.0
hsa_miR_451	318.0
hsa_miR_133b	285.5
hsa_miR_29b	268.0
hsa_miR_92	246.0
hsa_miR_422b	236.5
hsa_miR_1	229.5
hsa_miR_15a	222.0
hsa_miR_196b	211.0
hsa_miR_93	210.5
hsa_miR_128b	209.0
hsa_miR_494	202.5
hsa_miR_361	202.0
hsa_miR_199b	196.0
hsa_miR_223	194.5
hsa_miR_98	192.0
hsa_miR_25	186.0
hsa_miR_200a	185.0
hsa_miR_106b	184.5
hsa_miR_28	184.5
hsa_miR_146a	179.5
hsa_miR_29c	175.0
hsa_miR_146b	170.5
hsa_miR_181a	155.0
hsa_miR_150	153.0
hsa_miR_424	142.0
hsa_miR_495	142.0
hsa_miR_187	136.5
hsa_miR_128a	129.5

hsa_miR_218	121.5
hsa_miR_151	113.5
hsa_miR_20b	113.5
hsa_miR_503	112.0
hsa_miR_379	111.0
hsa_miR_181b	108.5
hsa_miR_224	108.0
hsa_miR_185	104.5
hsa_miR_141	104.0
hsa_miR_193b	103.0
hsa_miR_487b	98.0
hsa_miR_376a	96.0
hsa_miR_335	95.5
hsa_miR_455	94.5
hsa_miR_423	94.0
hsa_miR_20a	93.5
hsa_miR_452	77.0
hsa_miR_189	72.0
hsa_miR_194	67.5
hsa_miR_132	60.5
hsa_miR_126_AS	60.0
hsa_miR_331	56.5
hsa_miR_186	54.5
hsa_miR_130b	47.0
hsa_miR_139	44.0
hsa_miR_362	41.0
hsa_miR_140	40.5
hsa_miR_382	36.0
hsa_miR_429	35.5
hsa_miR_101	35.0
hsa_miR_210	31.5
hsa_miR_155	30.5
hsa_miR_204	30.0
hsa_miR_148b	28.5
hsa_miR_182	25.5
hsa_miR_324_3p	25.0
hsa_miR_181c	24.0
hsa_miR_432	24.0
hsa_miR_500	23.0
hsa_miR_17_3p	22.0
hsa_miR_345	22.0
hsa_miR_542_5p	21.5
hsa_miR_484	21.0
hsa_miR_181d	20.5
hsa_miR_192	20.0
hsa_miR_188	19.5

hsa_miR_363	19.5
hsa_miR_154	19.0
hsa_miR_422a	19.0
hsa_miR_134	18.5
hsa_miR_425	18.0
hsa_miR_491	18.0
hsa_miR_299_3p	17.5
hsa_miR_34b	17.5
hsa_miR_511	17.0
hsa_miR_505	16.5
hsa_miR_127	16.0
hsa_miR_142_5p	16.0
hsa_miR_299_5p	16.0
hsa_miR_381	16.0
hsa_miR_202	15.5
hsa_miR_377	15.5
hsa_miR_369_5p	14.5
hsa_miR_409_3p	14.0
hsa_miR_339	12.5
hsa_miR_30e_3p	11.5
hsa_miR_375	11.5
hsa_miR_493_3p	11.0
hsa_miR_539	10.5
hsa_miR_212	10.0
hsa_miR_376b	10.0
hsa_miR_198	9.5
hsa_miR_329	9.5
hsa_miR_378	9.5
hsa_miR_18a	8.5
hsa_miR_213	8.5
hsa_miR_34c	8.5
hsa_miR_409_5p	8.5
hsa_miR_452_AS	8.5
hsa_miR_9_AS	8.5
hsa_miR_149	8.0
hsa_miR_370	8.0
hsa_miR_502	8.0
hsa_miR_19a	7.5
hsa_miR_509	7.5
hsa_miR_197	7.0
hsa_miR_323	7.0
hsa_miR_365	7.0
hsa_miR_486	7.0
hsa_miR_95	7.0
hsa_miR_296	6.5
hsa_miR_30a_3p	6.5

hsa_miR_374	6.5
hsa_miR_513	6.5
hsa_miR_518c_AS	6.5
hsa_miR_18b	6.0
hsa_miR_363_AS	6.0
hsa_miR_373_AS	6.0
hsa_miR_136	5.5
hsa_miR_138	5.5
hsa_miR_206	5.5
hsa_miR_7	5.5
hsa_miR_340	5.0
hsa_miR_433	5.0
hsa_miR_517_AS	5.0
hsa_miR_193a	4.5
hsa_miR_196a	4.5
hsa_miR_492	4.5
hsa_miR_410	4.0
hsa_miR_488	4.0
hsa_miR_514	4.0
hsa_miR_518f	4.0
hsa_miR_520a	4.0
hsa_miR_523	3.5
hsa_miR_105	3.0
hsa_miR_18a_AS	3.0
hsa_miR_200a_AS	3.0
hsa_miR_301	3.0
hsa_miR_483	3.0
hsa_miR_487a	3.0
hsa_miR_489	3.0
hsa_miR_493_5p	3.0
hsa_miR_526a	3.0
hsa_miR_124a	2.5
hsa_miR_324_5p	2.5
hsa_miR_330	2.5
hsa_miR_518a_2_AS	2.5
hsa_miR_122a	2.0
hsa_miR_154_AS	2.0
hsa_miR_383	2.0
hsa_miR_501	2.0
hsa_miR_520d_AS	2.0
hsa_miR_302a_AS	1.5
hsa_miR_326	1.5
hsa_miR_371	1.5
hsa_miR_431	1.5
hsa_miR_485_3p	1.5
hsa_miR_517b	1.5

hsa_miR_96	1.5
hsa_miR_33	1.0
hsa_miR_346	1.0
hsa_miR_380_5p	1.0
hsa_miR_450	1.0
hsa_miR_453	1.0
hsa_miR_498	1.0
hsa_miR_508	1.0
hsa_miR_512_3p	1.0
hsa_miR_516_3p	1.0
hsa_miR_518d	1.0
hsa_miR_519a	1.0
hsa_miR_526b	1.0
hsa_miR_545	1.0
hsa_miR_142_3p	0.5
hsa_miR_211	0.5
hsa_miR_302a	0.5
hsa_miR_302c_AS	0.5
hsa_miR_373	0.5
hsa_miR_384	0.5
hsa_miR_449	0.5
hsa_miR_485_5p	0.5
hsa_miR_496	0.5
hsa_miR_504	0.5
hsa_miR_517a	0.5
hsa_miR_518a	0.5
hsa_miR_518c	0.5
hsa_miR_518e	0.5
hsa_miR_520c	0.5
hsa_miR_520f	0.5
hsa_miR_520h	0.5
hsa_miR_9	0.5
hsa_miR_129	0.0
hsa_miR_135a	0.0
hsa_miR_135b	0.0
hsa_miR_137	0.0
hsa_miR_144	0.0
hsa_miR_147	0.0
hsa_miR_153	0.0
hsa_miR_182_AS	0.0
hsa_miR_183	0.0
hsa_miR_184	0.0
hsa_miR_190	0.0
hsa_miR_191_AS	0.0
hsa_miR_202_AS	0.0
hsa_miR_208	0.0

hsa_miR_215	0.0
hsa_miR_216	0.0
hsa_miR_217	0.0
hsa_miR_219	0.0
hsa_miR_220	0.0
hsa_miR_302b	0.0
hsa_miR_302b_AS	0.0
hsa_miR_302c	0.0
hsa_miR_302d	0.0
hsa_miR_32	0.0
hsa_miR_325	0.0
hsa_miR_328	0.0
hsa_miR_337	0.0
hsa_miR_338	0.0
hsa_miR_367	0.0
hsa_miR_369_3p	0.0
hsa_miR_372	0.0
hsa_miR_376a_AS	0.0
hsa_miR_380_3p	0.0
hsa_miR_412	0.0
hsa_miR_432_AS	0.0
hsa_miR_448	0.0
hsa_miR_490	0.0
hsa_miR_499	0.0
hsa_miR_506	0.0
hsa_miR_507	0.0
hsa_miR_510	0.0
hsa_miR_512_5p	0.0
hsa_miR_515_3p	0.0
hsa_miR_515_5p	0.0
hsa_miR_516_5p	0.0
hsa_miR_517c	0.0
hsa_miR_518b	0.0
hsa_miR_518f_AS	0.0
hsa_miR_519b	0.0
hsa_miR_519c	0.0
hsa_miR_519d	0.0
hsa_miR_519e	0.0
hsa_miR_519e_AS	0.0
hsa_miR_520a_AS	0.0
hsa_miR_520b	0.0
hsa_miR_520d	0.0
hsa_miR_520e	0.0
hsa_miR_520g	0.0
hsa_miR_521	0.0
hsa_miR_522	0.0

hsa_miR_524	0.0
hsa_miR_524_AS	0.0
hsa_miR_525	0.0
hsa_miR_525_AS	0.0
hsa_miR_526b_AS	0.0
hsa_miR_526c	0.0
hsa_miR_527	0.0
hsa_miR_542_3p	0.0
hsa_miR_544	0.0

Supplementary Table 2. MiRNAs differentially expressed in individual HPV-16 positive cell lines compared to the normal cervix

MiRNA	CaSki Fold	SiHa Fold	20863 Fold	20861 Fold	201402 Fold
Overexpressed					
hsa_miR_141					10.11
hsa_miR_210					8.01
hsa_miR_31					7.81
hsa_miR_18a					7.80
hsa_miR_34c					7.37
hsa_miR_183		8.14			6.37
hsa_miR_136					5.66
hsa_miR_301					5.30
hsa_miR_182					5.18
hsa_miR_193a					4.82
Underexpressed					
hsa_miR_126				-26.24	-23.42
ambi_miR_7029					-13.66
hsa_miR_451				-12.05	-10.45
hsa_miR_133b					-10.24
hsa_miR_495				-8.16	-9.93
hsa_miR_146a					-8.71
hsa_miR_223					-8.36
hsa_miR_195			-18.17		-8.34
hsa_miR_218					-8.11
hsa_miR_133a			-10.12		-8.08
hsa_miR_199b		-19.52			-8.05
hsa_miR_1					-8.01
hsa_miR_497					-7.68
hsa_miR_487b					-7.21
hsa_miR_145	-51.55	-28.05	-35.46	-11.68	-7.02
hsa_miR_368			-16.60		-6.91
hsa_miR_150					-5.90
hsa_miR_382					-5.51
ambi_miR_7070					-4.98
hsa_miR_139					-4.80
hsa_miR_154					-4.63
hsa_miR_155					-4.23
hsa_miR_143		-22.61			
hsa_miR_199a			-14.74		
hsa_miR_214			-12.13		

The *q* value of all miRNAs was 0.

Supplementary Table 3. MiRNAs differentially expressed in the HPV-18 positive cell line HeLa compared to the normal cervix

MiRNA	HeLa Fold
Overexpressed	
ambi_miR_13143	320.8
hsa_miR_31	13.7
hsa_miR_106a	12.2
hsa_miR_93	8.9
ambi_miR_9630	7.1
hsa_miR_17_5p	6.6
hsa_miR_182	6.5
hsa_miR_196a	6.2
hsa_miR_30a_5p	5.6
hsa_miR_183	5.2
hsa_miR_25	4.5
hsa_miR_106b	4.4
hsa_miR_224	3.6
Underexpressed	
hsa_miR_1	-9.3
hsa_miR_145	-9.1
ambi_miR_7029	-8.4
hsa_miR_205	-8.0
hsa_miR_133b	-7.6
hsa_miR_143	-6.8
hsa_miR_214	-6.7
hsa_miR_199a	-5.7
hsa_miR_368	-5.6
hsa_miR_451	-5.3
hsa_miR_329	-4.6
hsa_miR_200a	-4.6
hsa_miR_200c	-3.7
hsa_miR_139	-3.3

The *q* value of all miRNAs was 0.

Supplementary Table 4. MiRNAs differentially expressed in individual HPV-16 positive cell lines compared to the HPV-negative cell line C-33A

MiRNA	CaSki Fold	SiHa Fold	20863 Fold	20861 Fold	201402 Fold
Overexpressed					
hsa_miR_205			50.15	24.19	46.30
hsa_miR_200c			36.05	21.68	33.59
hsa_miR_141			16.84	22.42	31.48
hsa_miR_203			14.69	16.42	30.52
hsa_miR_34a		8.08		9.19	16.64
ambi_miR_13258			7.98	9.34	13.14
hsa_miR_193b		55.41	10.59	8.65	10.82
hsa_miR_31		10.00	10.90		10.69
hsa_miR_200b			7.00	6.16	9.35
hsa_miR_200a			5.35		8.48
hsa_miR_503		6.02		5.54	7.25
hsa_miR_224			5.12	5.94	7.16
hsa_miR_199a					6.85
hsa_miR_199a_AS					6.63
hsa_miR_34c					6.53
hsa_miR_210		8.88			6.35
hsa_miR_27a		8.85			5.35
hsa_miR_29b					4.94
hsa_miR_193a					4.81
hsa_miR_27b		6.18			4.59
hsa_miR_422b					4.53
hsa_miR_34b					4.44
hsa_miR_145					4.24
hsa_miR_18a					4.05
hsa_miR_15a					3.93
hsa_miR_143					3.92
hsa_miR_136					3.54
ambi_miR_3046		29.34			
ambi_miR_12902		14.75			
hsa_miR_24		11.66			
hsa_miR_125a		10.86			
ambi_miR_13268		10.67			
hsa_miR_181b		10.42			
hsa_miR_99b		8.26			
hsa_miR_23b		7.58			
hsa_miR_23a		7.26			
hsa_miR_152		6.90			
Underexpressed					
hsa_miR_99a	-19.37				

hsa_miR_138				-8.50	

The *q* value of all miRNAs was 0.

Supplementary Table 5. MiRNAs differentially expressed in the HPV-18 positive cell line HeLa compared to C-33A

MiRNA	HeLa Fold
Overexpressed	
ambi_miR_13143	99.5
hsa_let_7i	16.5
hsa_miR_31	13.7
hsa_miR_34a	11.4
hsa_miR_193b	10.3
hsa_miR_224	7.7

The *q* value of all miRNAs was 0.

Supplementary Table 6. Probable targets of miRNAs. Computationally predicted targets were obtained from the miRBase Target database and cross-referenced with gene expression data obtained from Human Genome U133A 2.0 Arrays (Affymetrix)

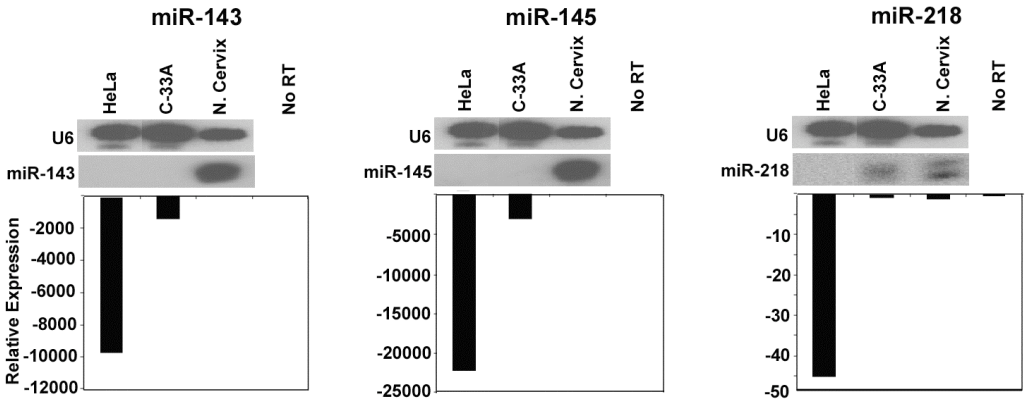
MiRNA	Number of targets	Probable Targets
miR-145	13	ADRM1, BAX, CENPA, DHCR7, GCSH, MRPS18B, NPM3, NUDT1, OGG1, POP5, SLC25A11, SNRPB, SSSCA1
miR-143	9	C1QBP, CDC2, F12, HMBS, SNRPB, SSBP1, STIP1, TP53, TUBB4
miR-368	13	BUB3, BYSL, HADH2, HRAS, ID1, KCNG1, MCM2, MT1X, NEFH, PLK1, SSBP1, TFAP2A, TPX2
miR-497	14	ABCF2, ARHGDIA, CHEK1, CLPP, FARSLA, HMBS, MRPL40, NDUFS6, OIP5, PMM1, POLR2E, PPIE, TUBA1, ZNHIT1
miR-218	17	ABCF2, DUSP5, EBP, EFEMP1, EFNA1, HSPA2, KRT6B, LAMB3, MBNL2, MRPS27, MT1G, NUP93, PLAUR, PPIE, RGS20, SPINT2, TPM1
miR-200c	16	APBB2, CNN3, CRYZ, DDAH1, DNAJC8, FKBP1B, GRSF1, GSTA4, HSPD1, MAGEA12, RGS5, SCG2, SLIT2, TAF12, TCF8, ZNF6

.

APPENDIX B

SUPPLEMENTARY FIGURES

Supplementary Figure 1. MiRNA expression data in HeLa, C-33A, and normal cervical tissue. For Northern blot analysis, the housekeeping splicing-related small U6 RNA was used as a loading control. For real-time qRT-PCR analysis, RNU43 served as the endogenous control for miRNAs.



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